EVIDENCE APPENDIX
EXHIBIT 16

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Identification and isolation of multipotential neural progenitor cells from the subcortical white matter of the adult human brain

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The subcortical white matter of the adult human brain harbors a pool of glial progenitor cells. These cells can be isolated by fluorescence-activated cell sorting (FACS) after either transfection with green fluorescent protein (GFP) under the control of the *CNP2* promoter, or A2B5-targeted immunotagging. Although these cells give rise largely to oligodendrocytes, in low-density culture we observed that some also generated neurons. We thus asked whether these nominally glial progenitors might include multipotential progenitor cells capable of neurogenesis. We found that adult human white-matter progenitor cells (WMPCs) could be passaged as neurospheres *in vitro* and that these cells generated functionally competent neurons and glia both *in vitro* and after xenograft to the fetal rat brain. WMPCs were able to produce neurons after their initial isolation and did not require *in vitro* expansion or reprogramming to do so. These experiments indicate that an abundant pool of mitotically competent neurogenic progenitor cells resides in the adult human white matter.

The adult human subcortical white matter harbors a population of mitotically competent glial progenitors that comprise as many as 3% of its cells[1,2]. These cells may be extracted from brain tissue using FACS after transfection with GFP-encoding plasmids driven by the promoter for *CNP*, an early oligodendrocytic transcript[2,3]. The cells express the immature neural ganglioside recognized by monoclonal antibody A2B5 but do not express more mature markers of glial lineage. We previously noted that when grown at high density, pCNP2:hGFP⁺ progenitors gave rise to glia, largely oligodendrocytes. Nonetheless, in low-density culture after high-purity FACS, pCNP2:hGFP⁺ cells often generated βIII-tubulin⁺ neurons[2]. Because neurogenesis was never observed from pCNP2:hGFP⁺ cells in higher-density or unsorted cultures, we postulated that the restriction of these progenitor cells to the oligodendroglial phenotype might be an effect of environmental cues rather than a function of autonomous commitment. Once isolated into high-purity, low-density culture, and therefore removed from any paracrine or autocrine influences, human subcortical pCNP2:hGFP⁺ cells were able to generate neurons as well as glia[2]. It was subsequently reported[4] that glial progenitors from the postnatal rat optic nerve could also generate neurons after serum- or bone morphogenetic protein–induced phenotypic instruction and basic fibroblast growth factor (bFGF)–stimulated expansion. Similar work showed that progenitor cells of the adult rat forebrain parenchyma could also generate neurons after prolonged *in vitro* expansion in bFGF[5]. Taken to-

gether, these findings indicated that glial progenitor cells might retain substantial phenotypic plasticity.

We asked whether some fraction of the nominally glial progenitors of the adult human subcortical white matter might actually be parenchymal neural stem cells. Specifically, we asked whether single, sorted WMPCs could generate multiple neural phenotypes, and if so, whether they were capable of expansion and self-renewal. In addition, we investigated whether this process requires de-differentiative reprogramming to an intermediate phenotype, or whether simply removing these cells from their local environment and mitotically expanding them in bFGF might suffice to permit these cells to act as multipotential progenitors. In doing so, we tested the hypothesis that the phenotypic plasticity of adult WMPCs might be tonically restricted by the adult parenchymal environment, rather than irreversibly lost with development.

WMPCs were isolated by *CNP*- and A2B5-based sorting

White matter was dissected from surgical samples taken at the time of temporal lobectomy for epilepsy, aneurysm, and post-traumatic decompression (*n* = 21). The tissues were dissected free of adjacent cortex and ventricular epithelium, and enzymatically dissociated to single-cell suspension as described[2]. The dissociates were plated onto laminin (100 µg/ml) in DMEM/F12/N1 supplemented with bFGF (20 ng/ml), NT3 (2 ng/ml) and platelet-derived growth factor (PDGF)-AA (20 ng/ml). To identify oligodendrocyte progenitors, the dissociates were trans-

fected with pCNP2:hGFP, the transcription of which results in GFP expression by oligodendrocyte progenitor cells[2].

To avoid both the temporal lag between transfection and GFP expression and the inefficiency of plasmid transfection, cultures were also sorted on the basis of A2B5 surface immunoreactivity, which can serve as a surrogate marker for pCNP2:hGFP[+] WMPCs *in vitro*[2]. Immunostaining showed that 84 ± 8.3% of pCNP2:GFP^{+} cells expressed A2B5 (ref. 2). GFP-based FACS gated 0.49 ± 0.15% of all white-matter cells as pCNP2:hGFP[+] (mean ± s.e.m.; *n* = 3 patients; Fig. 1a). Matched cultures transfected with pCMV:GFP had a net transfection efficiency of 13.1%. Thus, the predicted incidence of pCNP2:hGFP^{+} cells in the white matter was 3.7% (= 1 + 0.131 × 0.49), consistent with our prior estimates of the incidence of this phenotype[2]. From the same samples, A2B5-based FACS gated an average of 3.1 ± 0.7% (*n* = 3) of the white-matter cell population (Fig. 1b). The greater than six-fold increase in net yield when A2B5 was used (3.1% versus 0.49%) reflected the higher efficiency of A2B5 immunodetection relative to pCNP2:hGFP plasmid transfection. On this basis, we used immunomag-

netic sorting (IMS) to select A2B5^{+} cells from adult white-matter dissociates. By IMS, the incidence of A2B5-sorted cells in white matter dissociates was 3.6 ± 0.3% (*n* = 21) with a median of 3.1%. This improved yield was accomplished with no appreciable loss of cell-type specificity, in that the A2B5^{+} cells overlapped entirely with the sort profiles of pCNP2:hGFP^{+} cells and each isolate generated O4[+] oligodendrocytes with similar efficiency (Fig. 1c–f). Thus, A2B5-based FACS and IMS identified WMPCs homologous to those recognized by pCNP2:GFP-based FACS, while permitting higher-yield isolation of these cells.

Adult WMPCs gave rise to multipotent neurospheres

To assess the expansion capacity of pCNP2:hGFP- and A2B5-sorted cells, we propagated sorted isolates of each in suspension[4-8]. The cells were distributed into 24-well plates at 50,000 cells per 0.5 ml in serum-free media (SFM) supplemented with bFGF (20 ng/ml), NT3 (2 ng/ml) and PDGF-AA (20 ng/ml), a combination that permits the expansion of human WMPCs[2]. Seven days later, the cells were switched to SFM with bFGF alone (20 ng/ml)[8]. Over the next 10 d, neurospheres—spherical masses of cells that expand from single parental progenitors—arose in these cultures, such that by 3 weeks after sorting, there were 84.8 ± 9.0 spheres/well (*n* = 4 patients). These neurospheres were typically >150 μm in diameter and included 46.5 ± 8.2 cells/sphere (Fig. 2a and b). Thus, single WMPCs of the adult human brain were capable of generating neurospheres.

To establish the lineage potential of single adult human WMPCs, we dissociated the resultant primary neurospheres and passaged them into new wells. Alternatively, some were plated onto substrate to permit their differentiation. Immunostaining showed that both pCNP2:hGFP[+] and A2B5[+] progenitor–derived spheres gave rise to all major neural phenotypes (Fig. 2d and e). Among those cells passaged from primary spheres, secondary spheres were observed to arise within two weeks after passage. After expansion, these secondary spheres were similarly plated on substrate, raised for one to two weeks and fixed. Immunolabeling confirmed that virtually all secondary spheres generated both neurons and glia together (Fig. 2c and e). In addition, when the mitotic marker BrdU was added to A2B5-sorted cells, BrdU-incorporating neurons, oligodendrocytes and astrocytes all emerged from the spheres generated (Fig. 2f–i). The persistence of mitotic neurogenesis and gliogenesis by single spheres indicated that they contained cycling multipotential cells. The secondary spheres were probably of clonal origin, given the low plating density of the single cells from which each was derived and the fact that the sphere-forming cells originated from primary spheres that had themselves expanded from single-cell dissociates. These data indicate that single progenitor cells of the adult human white matter are both clonogenic and multipotent.
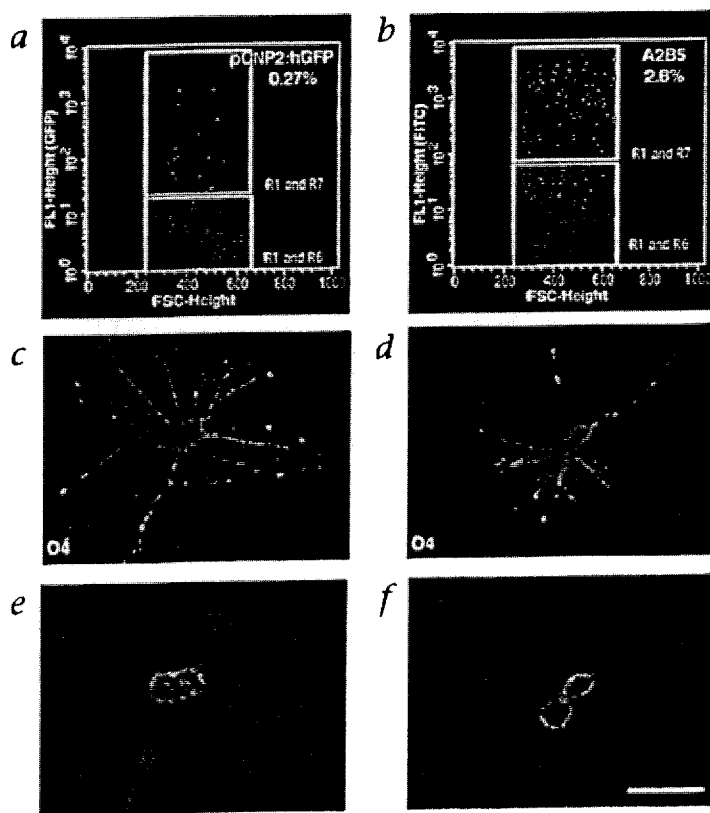

Fig. 1 A2B5-based FACS selects oligodendrocyte progenitor cells. *a* and *b*, FACS graphs showing the extraction of pCNP2:hGFP[+] (*a*) and A2B5[+] (*b*) WMPCs from an adult human white-matter dissociate. Forward scatter (FCS), a measure of cell size, is plotted against fluorescence intensity (FI-1). When pCNP2:hGFP- and A2B5-based sorts were directly compared, their plots showed overlapping profiles, but A2B5^{+} cells were >6-fold more abundant than their pCNP2:hGFP[+] counterparts, reflecting the higher efficiency of A2B5 surface tagging. *c–f*, Progenitors sorted by pCNP2:hGFP (*c* and *e*) and A2B5 (*d* and *f*) gave rise to O4[+] oligodendrocytes. A2B5-based surface antigen sorting may thus be used as a higher-yield alternative to pCNP2:hGFP transfection–based FACS for isolating WMPCs. Scale bar, 24 μm.
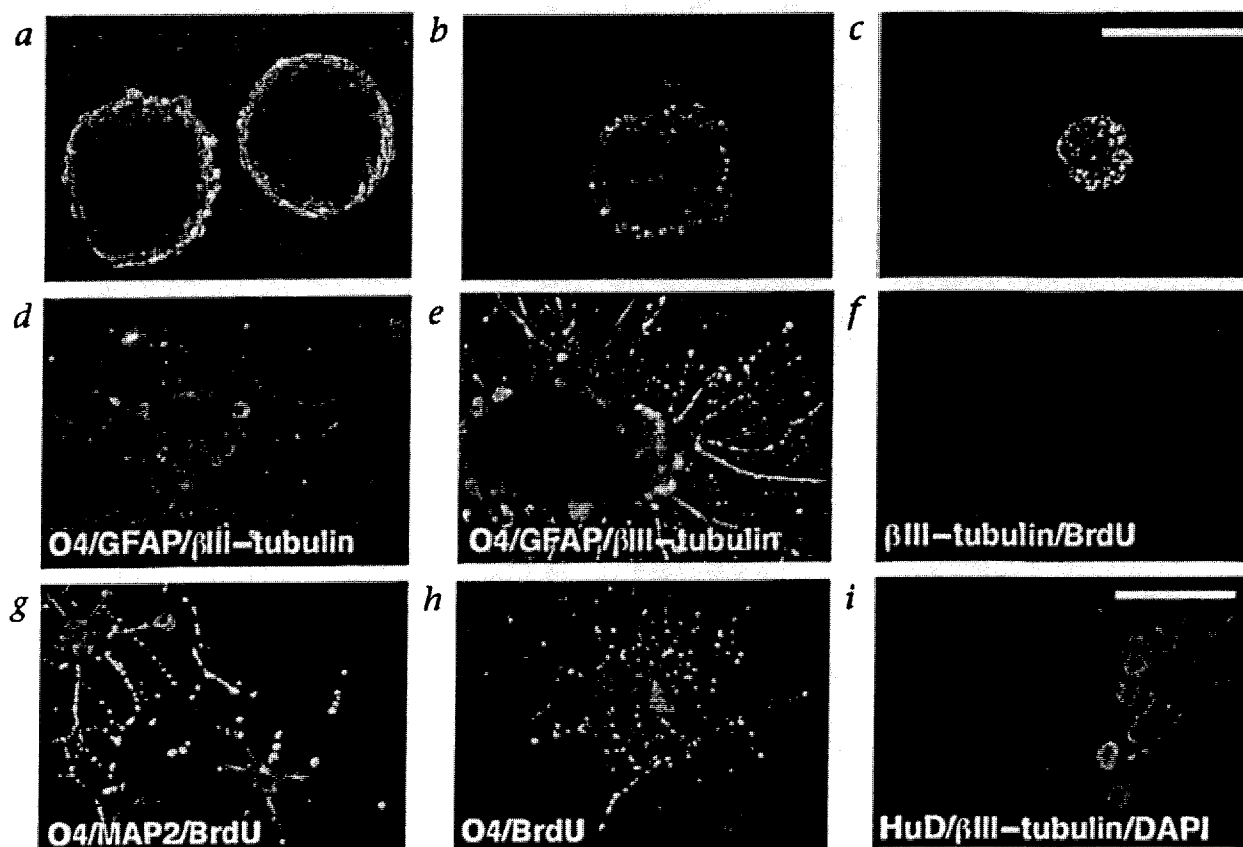
Fig. 2 Adult human WMPCs give rise to multipotential neurospheres. *a*, First-passage spheres generated from A2B5-sorted cells 2 weeks after sorting. *b*, First-passage spheres arising from pCNP2:hGFP-sorted cells, at 2 weeks. *c*, Second-passage sphere derived from an A2B5-sorted sample, at 3 weeks. *d*, Once plated onto substrate, the primary spheres differentiated into βIII-tubulin⁺ neurons (red), GFAP⁺ astrocytes (blue) and O4⁺ oligodendrocytes (green). *e*, Neurons (red), astrocytes (blue) and oligo-dendrocytes (green) arose similarly from spheres derived from pCNP2:GFP-sorted WMPCs. *f–h*, BrdU incorporation (blue) showed that new neurons (*f*, βIII-tubulin (red); *g*, MAP2 (red)) and oligodendrocytes (*h*, O4 (green)) were generated *in vitro*. *i*, βIII-tubulin⁺ neurons (green) co-expressed neuronal Hu protein⁴⁵⁴¹ (red), yielding double labeling (yellow). Nuclei were counterstained with DAPI (blue). Scale bars, 100 µm (*a–e*) or 40 µm (*f–i*).

Single WMPCs remained multipotential with passage

The serial propagability of sorted WMPCs from neurospheres in low-density dissociates suggested the clonal derivation of each individual sphere⁹⁻¹¹. To further validate the clonal origin of neurons and glia arising within single spheres, we used lentiviral GFP to genetically tag and follow single WMPCs. A2B5⁺ cells were tagged, 2–5 d after sorting, with a lentivirus expressing GFP under cytomegalovirus (CMV) promoter control¹²⁻¹⁴. At 10 PFU/cell, 23% of the cells expressed GFP by one week after sorting, yielding a mixture of GFP⁻, GFP⁺ and mixed spheres in the resultant cultures (Fig. 3*a–b*). These primary spheres were triturated two to four weeks later to single-cell suspensions and passaged into bFGF at ~3,000 cells/well. Under these conditions, 40.8 ± 12.9 secondary spheres/well were generated, indicating a clonogenic cell incidence of 1.3% (*n* = 5). Of these secondary spheres, 47.2 ± 10.8% contained only GFP⁺ cells (Fig. 3*c–d*) whereas 30.9 ± 6.9% harbored no GFP⁺ cells. The relative uniformity of GFP expression, or lack thereof, among the cells within a given sphere indicated that most spheres were clonally derived (*P* < 0.005 by χ² analysis). This tested the null hypothesis that the spheres arose from non-clonal aggregation of two or more cells, each of which was equally likely to be GFP⁺ or GFP⁻. When the single spheres were plated onto polyornithine and fibronectin and their outgrowth assessed two weeks later, all gave rise to both neurons and glia (Fig. 3*e–g*). Because most secondary spheres were likely to have been clonally derived, and all included neurons as well as glia (38 of 38 spheres; *n* = 4 samples), single WMPCs must have given rise to neurons and glia together.

We next asked if the neurogenic capacity and multilineage competence of WMPCs were maintained with passage. Primary spheres were raised serially in bFGF/NT3/PDGF-AA for 7 d, DMEM/F12/N1 with 15% serum/PDGF-AA for 4 d, and serum-free DMEM/F12/N1 with bFGF for 10 d. Cells were then dissociated and replated in bFGF at 3,000 cells/well in a 24-well plate. Secondary spheres arose within two weeks from 1.1 ± 0.3% of these cells (*n* = 8). After more than two weeks of further expansion, the secondary spheres were plated on polyornithine and fibronectin and were fixed and immunostained two weeks later (seven to nine weeks after sorting). Whereas primary spheres consisted of 21.7 ± 4.3% βIII-tubulin⁺ neurons, 17.7 ± 3.9% glial fibrillary acidic protein (GFAP)⁺ astrocytes and 46.7 ± 5.9% O4⁺ oligodendrocytes (*n* = 3), secondary spheres consisted of 16.0 ± 2.5% neurons, 19.3 ± 3.2% astro-

cytes and 46.4 ± 2.4% oligodendrocytes ($n = 3$). Most of the neurons were GABAergic, by virtue of their expression of glutamic acid decarboxylase-67 (GAD67) (Fig. 4a–c). Because the relative proportions of neurons, oligodendrocytes and astrocytes in secondary spheres were similar to those in primary spheres, we concluded that WMPCs retained multilineage competence with expansion.

WMPC-derived neurons become functionally mature

The calcium responses and membrane currents of WMPC-derived neurons were assessed to establish their ability to respond to depolarizing stimuli. Primary spheres ($n = 12$ fields, derived from 3 brains) were plated on fibronectin to permit neuronal outgrowth, and assessed 14 d later for their calcium responses to depolarizing stimuli. The cultures were then loaded with the calcium indicator dye Fluo-3 and serially exposed to both 100 µM glutamate and 60 mM potassium during confocal microscopy. Astrocytic responses to depolarization were minimal under these culture conditions, as previously noted. In contrast, neuron-like cells displayed rapid, reversible, >100% elevations in cytosolic calcium in response to potassium, consistent with the activity of neuronal voltage-gated calcium channels (Fig. 4d–f). The neuronal phenotype of these cells was then validated by immunostaining for βIII-tubulin.

We then asked whether WMPC-derived neurons would be able to develop the fast sodium currents and action potentials characteristic of electrophysiologically competent neurons. We used whole-cell patch-clamp recording during current stimulation to assess the response of WMPC-derived neurons that arose from plated secondary spheres derived from A2B5-

sorted isolates. A total of 58 WMPC-derived fiber-bearing cells were recorded, in 5 cultures derived from 3 patients. Of these, 13 showed voltage-activated sodium ion currents (I_{Na}) of >100 nA, and 7 had $I_{Na} > 600$, compatible with the fast sodium currents of neuronal depolarization[1b,14]. Accordingly, whereas two of five cells with $I_{Na} > 800$ generated stimulus-evoked action potentials (Fig. 4g–h), none did so with $I_{Na} < 800$. In addition, none of 26 morphologically non-neuronal cells showed substantial (≥100 pA) current-induced sodium currents. Together, these results indicated that neurons arising from adult human WMPCs developed mature electrophysiologic functions, including both fast sodium currents and action potentials.

WMPCs generated neurons without reprogramming

Glial progenitor cells from the postnatal rat optic nerve can generate neurons, under conditions that have been described as 'reprogramming' glial progenitors to multilineage competence[4]. In that study, neurogenesis was achieved by first instructing the cells to an intermediary astrocytic lineage using either serum or bone morphogenetic protein-2, followed by bFGF-stimulated mitogenesis. We asked whether such reprogramming steps are required for the generation of neurons from adult human WMPCs, or whether simple expansion under minimal conditions *in vitro*, with the removal of these cells from their environment, might be sufficient to permit neurogenesis by these cells. Sorted A2B5+ cells were cultured in several permutations of mitogenic and differentiative conditions to identify the minimal conditions permissive for lineage diversification. We compared the phenotypes generated under three conditions: (i) bFGF/NT3/PDGF-AA in SFM (composed of DMEM/F12/N1) for 7 d, followed by 15% FBS/ PDGF-
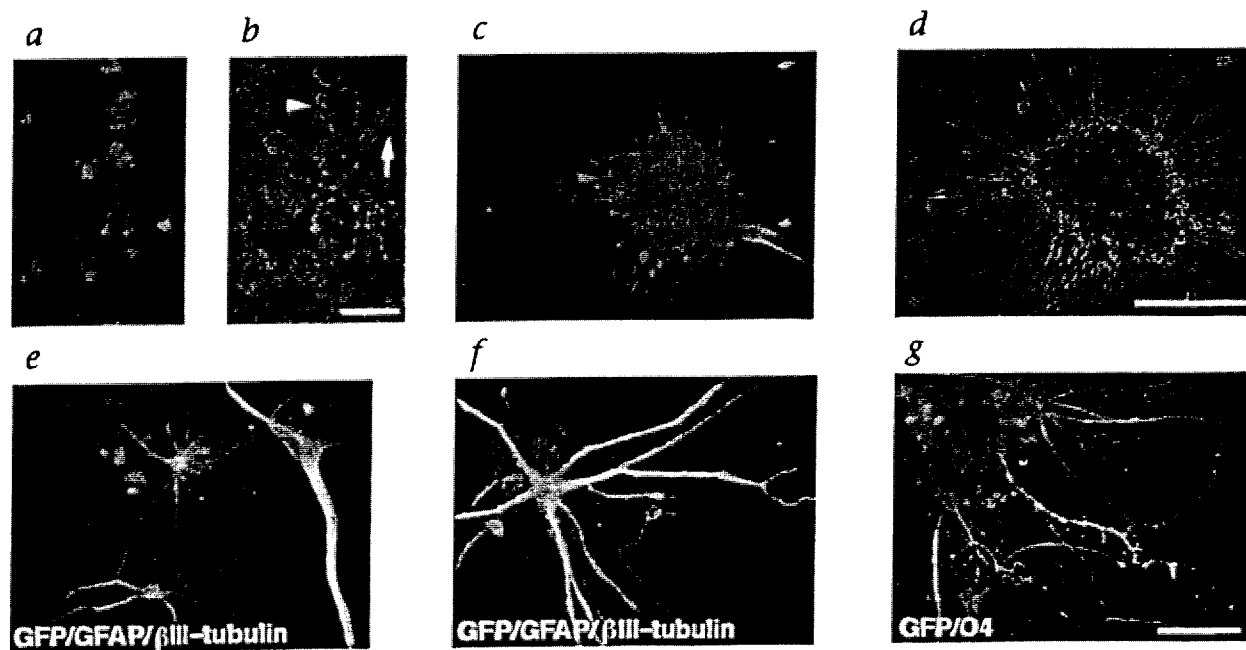

Fig. 3 Single lentiviral GFP-tagged WMPCs generated neurons and glia. A2B5-sorted WMPCs were infected with a lentivirus encoding enhanced GFP[14], 5 d after sorting. *a* and *b*, Secondary spheres subsequently derived from infected cells harbored either GFP-tagged cells (arrowhead), untagged cells (arrow) or, less commonly, both. *c* and *d*, GFP+ secondary sphere 1 week after plating. *e* and *f*, βIII-tubulin+ neurons (red) and GFAP+ astrocytes (blue) arising from a single clonally derived GFP+ secondary sphere. *g*, GFP+ (green) and O4+ (red) oligodendrocytes arising from a secondary sphere. Scale bars, 100 µm (*a* and *b*), 60 µm (*c* and *d*) or 40 µm (*e–g*).

AA for 4 d and SFM with bFGF for two weeks; (ii) bFGF/NT3/PDGF-AA in SFM continuously for three weeks; and (iii) bFGF alone in SFM for three weeks. The first condition was intended to promote initial differentiation in serum, whereas the latter two groups were designed to skip this glial differentiative step[4].

The A2B5-sorted progenitors yielded spheres under each of these conditions; however, both the number of spheres and the percentage of neurons generated by each differed as a function of treatment. Cultures maintained in base media alone or in bFGF-supplemented media had $5.9 \pm 1.7\%$ and $7.2 \pm 2.1\%$ βIII-tubulin* neurons, respectively ($n = 3$ patients). When matched WMPC-derived spheres were sequentially raised in bFGF/NT3/PDGF-AA with 15% serum and bFGF, $18.2 \pm 2.2\%$ of the cells were βIII-tubulin* (Fig. 5a). A similar proportion of neurons ($22.5 \pm 1.9\%$; $n = 3$) was generated by those neurospheres maintained in SFM with bFGF/NT3/PDGF-AA. Serum exposure was therefore not required for A2B5* cells to generate neurons. Indeed, no specific signals seemed necessary for neuronal instruction, besides those provided by PDGF and NT3. These data indicated that antecedent astrocytic differentiation was not a necessary prerequisite to neurogenesis by adult WMPCs. These cells required neither prolonged mitogenic expansion, nor specific dedifferentiation steps, to generate neurons as well as glia[4].

Although both PDGF and NT3 promote oligodendrocyte production by glial progenitors of the rat optic nerve[17,18], each can induce neuronal differentiation in less-committed hippocampal and ventricular zone neural progenitors[19,20]. As such, their neurogenic effects on adult WMPCs may reflect the relatively undifferentiated state of these cells.

Fig. 4 WMPC-derived neurons showed functional maturation *in vitro*. *a–c*, Neurons derived from adult human WMPCs had a GABAergic phenotype. *a*, Outgrowth of a WMPC-derived neurosphere, stained for neuronal βIII-tubulin after 35 d *in vitro*. *b*, Immunostaining showed that all 9 neurons in the field were GAD67* and were thus likely to be GABAergic. *c*, DAPI nuclear counterstaining showed the abundance of cells in the field. *d–f*, WMPC-derived neurons developed neuronal Ca²⁺ responses to depolarization. *d*, WMPC-derived cells loaded with the calcium indicator dye Fluo-3, 10 d after plating of first-passage spheres derived from A2B5-sorted white matter (35 d *in vitro* total). Many fiber-bearing cells of both neuronal and glial morphologies are apparent. *e*, The same field after exposure to 100 μM glutamate. *f*, The same field after exposure to a depolarizing stimulus of 60 mM KCl. Rapid, reversible, >100% elevations in cytosolic calcium occurred in response to K*, consistent with the activity of neuronal voltage-gated calcium channels. Scale bar, 80 μm. *g* and *h*, Whole-cell patch-clamp experiments detected voltage-gated sodium currents and action potentials in WMPC-derived neurons. *g*, Representative cell, 14 d after plating of first-passage sphere derived from A2B5-sorted white matter. The cell was patch clamped in a voltage-clamped configuration and its responses to current injection were recorded. *h*, Action potentials (AP) were noted after positive current injection, at I_{in} >800 pA (left tracing). The fast negative deflections noted after depolarization steps are typical of the voltage-gated sodium currents of mature neurons (right).

Only a fraction of A2B5⁻ cells were clonogenic

We next assessed the incidence of clonogenic and multipotential progenitor cells within the larger pool of A2B5-sorted white-matter cells. We first assessed whether either the survival or the mitotic competence of adult human WMPCs were dependent on mitotic competence of adult human WMPCs were dependent on mitotic density, by assessing the limiting dilution at which clonogenic progenitors could be obtained from A2B5-sorted white-matter dissociates. A2B5* cells were plated immediately after sorting, at densities ranging from 100,000 to 1,000 cells/ml (0.5 ml cell suspension per well of a 24-well plate), in basal media supple-

mented with bFGF/NT-3/PDGF-AA. Under these conditions, the incidence of clonogenic progenitors was a curvilinear function of the sorted cell density ($R^2 = 0.978$; Fig. 5b). Whereas 186 ± 7.6 spheres were generated at a density of 100,000 cells/ml (0.4%; $n = 5$ patients), only 6.5 ± 2.7 were noted at 10,000 cells/ml (0.1%) and no sphere generation was noted at or below 5,000 cells/ml. Thus, the expansion of purified WMPCs was density dependent and optimal at 50,000–100,000 cells/ml. Densities higher than the optimal range seemed to promote terminal differentiation of the progenitors.
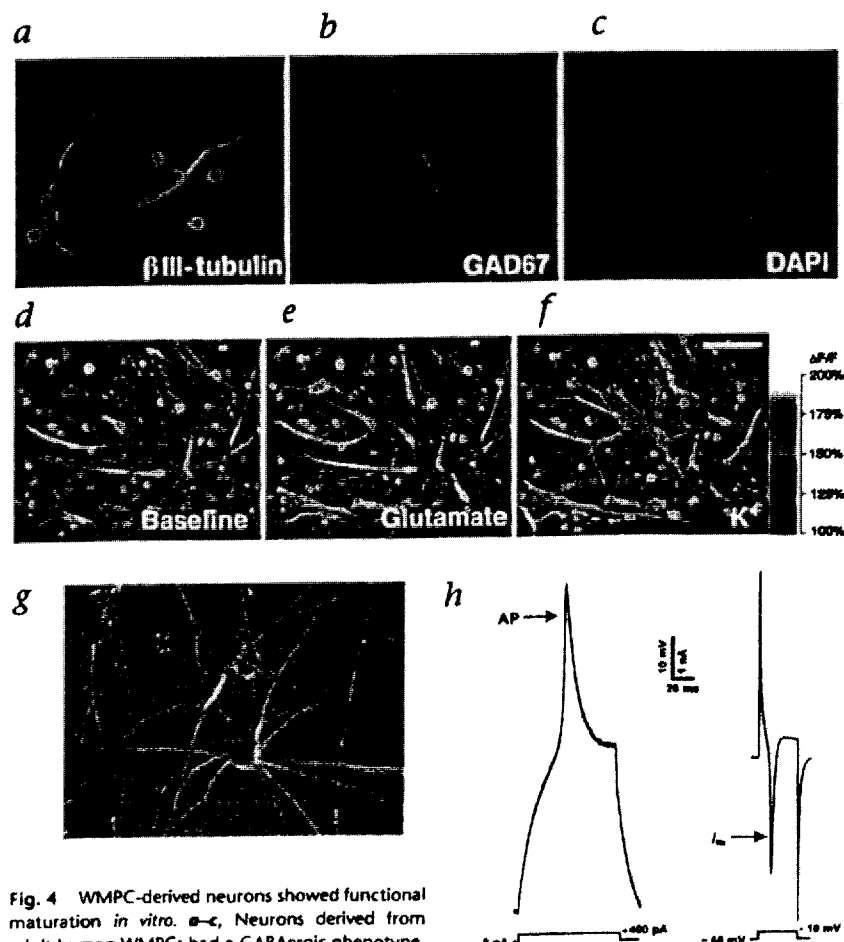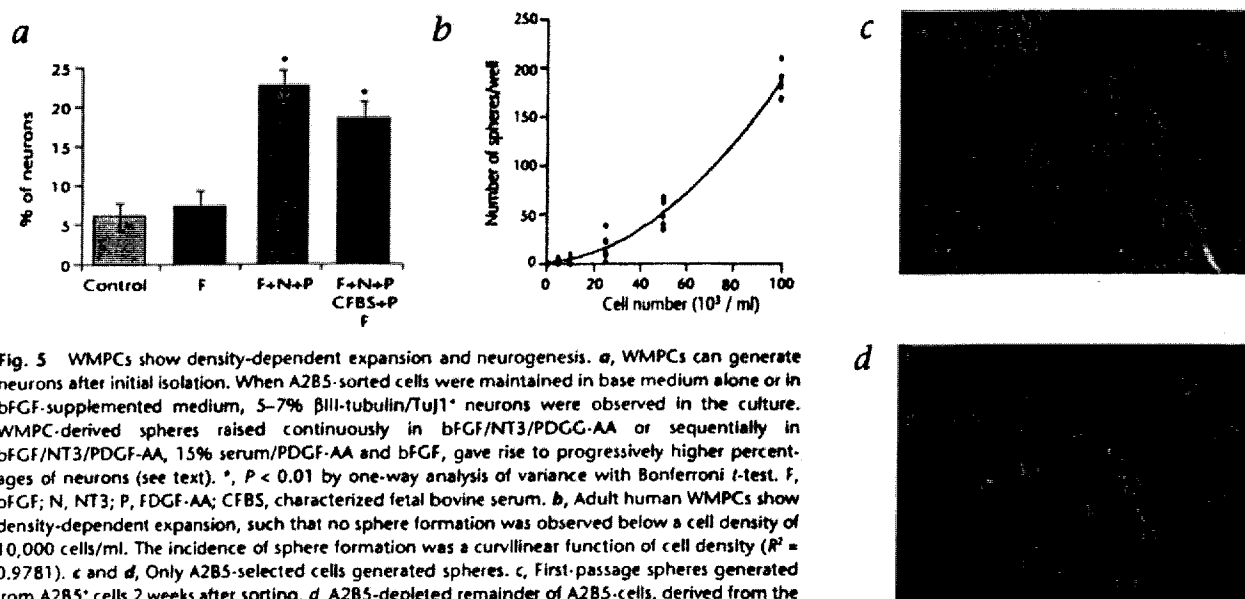
Fig. 5 WMPCs show density-dependent expansion and neurogenesis. *a*, WMPCs can generate neurons after initial isolation. When A2B5-sorted cells were maintained in base medium alone or in bFGF-supplemented medium, 5–7% βIII-tubulin/TuJ1⁺ neurons were observed in the culture. WMPC-derived spheres raised continuously in bFGF/NT3/PDGG-AA or sequentially in bFGF/NT3/PDGF-AA, 15% serum/PDGF-AA and bFGF, gave rise to progressively higher percentages of neurons (see text). *, P < 0.01 by one-way analysis of variance with Bonferroni t-test. F, bFGF; N, NT3; P, PDGF-AA; CFBS, characterized fetal bovine serum. *b*, Adult human WMPCs show density-dependent expansion, such that no sphere formation was observed below a cell density of 10,000 cells/ml. The incidence of sphere formation was a curvilinear function of cell density (R^2 = 0.9781). *c* and *d*, Only A2B5-selected cells generated spheres. *c*, First-passage spheres generated from A2B5⁺ cells 2 weeks after sorting. *d*, A2B5-depleted remainder of A2B5-cells, derived from the same source culture as cells in (*c*), exhibited no evidence of sphere formation 2 weeks after sorting.

To assess whether clonogenic WMPCs were restricted to the A2B5⁺ population, we also cultured the A2B5-depleted pool remaining after each sort. A2B5-depleted cultures did not give rise to any passageable neurospheres at any of the cell densities assessed over the range of 1,000–100,000 cells/ml (Fig. 5*d*). On the basis of these studies, we concluded that only a fraction of white-matter A2B5⁺ cells are actually clonogenic and multipotential progenitors, although all clonogenic WMPCs are A2B5⁺.

Adult WMPCs showed limited self-renewal

We next sought to define the extent to which WMPCs were self-renewing by assessing the extent to which WMPC-derived neurospheres were capable of repetitive passage. Primary spheres were raised from three patients at an optimal initial density of 100,000 cells/ml, under the conditions identified as most supportive of multilineage expansion (bFGF/NT3/PDGF-AA in DMEM/F12/N1). One month later, the spheres were dissociated and replated. Secondary spheres were generated and were replated one month later at 1 × 10⁴–5 × 10⁴ cells/ml. These cultures gave rise to tertiary spheres over the following month, though with less efficiency and a smaller volumetric expansion than secondary spheres. Attempts at propagating these spheres as quaternary spheres, after additional dissociation, were generally unsuccessful. Given an apparent cell doubling time of 3–4 d (data not shown) and monthly passages spanning 8–10 doublings, we estimated that the tertiary spheres assessed one month after the last passage underwent a minimum of 16–24 and no more than 30 doublings. This is well below the number of doublings of which tissue-derived stem cells are typically thought capable.

Our inability to successfully passage these cells beyond 16–24 doublings called into question their ability to self-replicate for extended periods of time *in vitro*. Their limited replicative competence contrasted with that of neural progenitors sorted from the fetal human ventricular zone, which may be readily passaged for >60 doublings under analogous culture conditions[31].

Such self-renewal capacity has been ascribed to sustained telomerase activity in a number of developing systems, including the fetal human forebrain[22,23]. To assess whether the apparently finite proliferative potential of adult human WMPCs reflected a lack of telomerase activity, telomerase levels were assessed using the telomerase reverse transcriptase activity protocol (TRAP) assay[23,24]. We did not detect any telomerase activity in primary or secondary WMPC-derived spheres, despite high-level activity in a variety of positive controls (see Supplementary Fig. 1 online). Their lack of extended replicative potential, coupled with their lack of telomerase activity, suggests that adult WMPCs might constitute a pool of multipotential progenitors with a finite capacity for mitotic expansion, transitional between tissue-restricted stem cells and phenotypically committed progenitors.

WMPCs produced neurons and glia after fetal xenograft

We next assessed whether WMPCs were multipotential *in vivo* as well as *in vitro* by evaluating their fate after engraftment to embryonic stage (E)17 fetal rat brains. Some A2B5-sorted cells were transplanted 24–48 h after sorting to assess their lineage potential upon initial isolation. These cells were maintained only in SFM during the period between isolation and xenograft and were never exposed to any exogenous growth factors. Other cells were transplanted 10 d after sorting, after maintenance in bFGF/NT3/PDGF-AA for 4 d and 15% serum/PDGF-AA followed by bFGF, for 3 days each. All donor cells were administered into E17 rat embryos by intraventricular injection at 10⁵ cells/animal. The recipients were killed and fixed four weeks after birth to evaluate the fate of the implanted human cells. Human donor cells were identified by immunolabeling of brain sections for human nuclear antigen (HNA).

In rats implanted with propagated WMPCs (Fig. 6) and their counterparts injected with acutely isolated WMPCs (see Supplementary Fig. 2 online), donor-derived migrants co-expressing HNA with either nestin or doublecortin[25] were found in the host olfactory subpendyma and hippocampus (Fig. 6*a* and
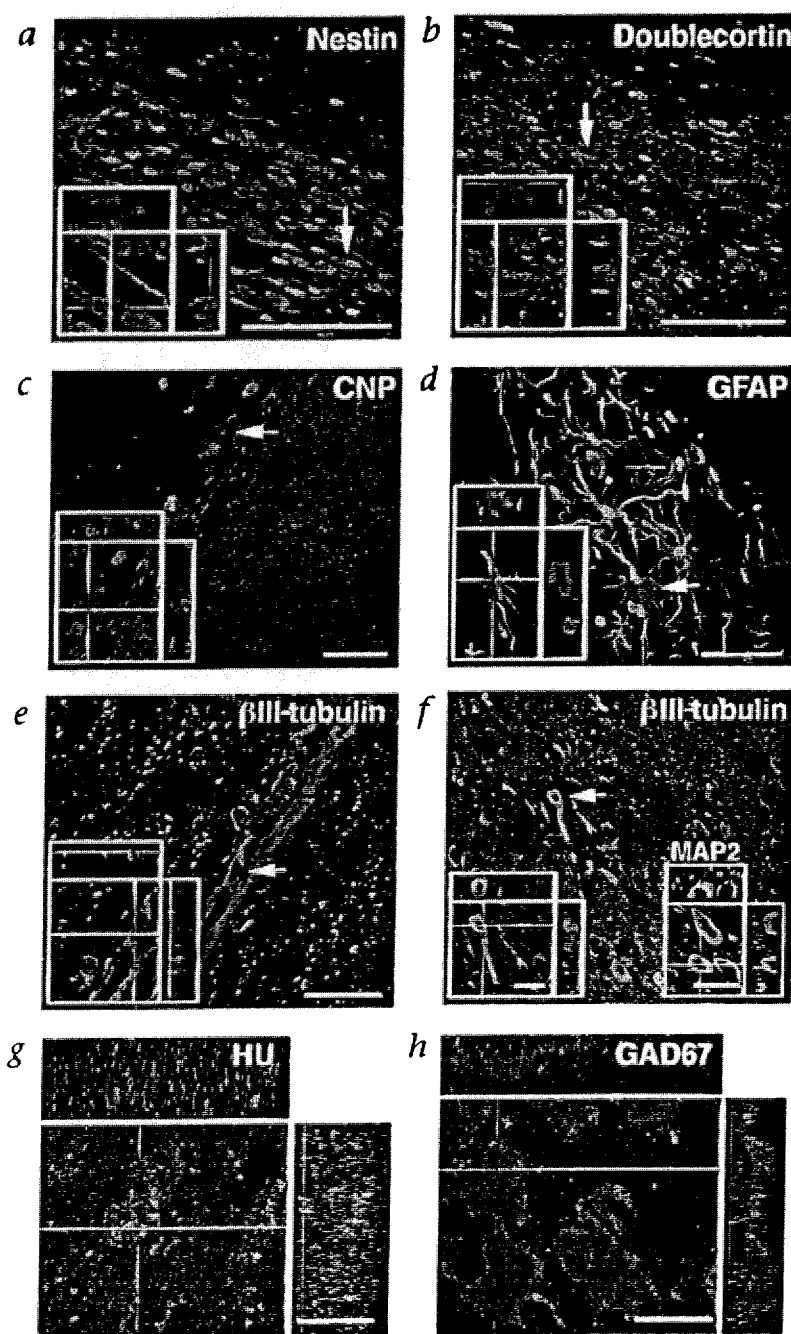
Fig. 6 WMPCs engrafted into fetal rats give rise to neurons and glia in a site-specific manner. Sections from a rat brain implanted at E17 with A2B5-sorted WMPCs and killed 1 month after birth. Cells were maintained in culture for 10 d before implanting. *a* and *b*, Nestin⁺ (*a*) progenitors and doublecortin⁺ (*b*) migrants (red) each co-expressing HNA (green) in the hippocampal alvius. *c*, CNP⁺ (red) HNA⁺ (green) oligodendrocytes, found exclusively in the corpus callosum. *d*, Low-power image of GFAP⁺ (green) HNA⁺ (red) astrocytes (yellow, double-positive) along the ventricular wall. *e*, βIII-tubulin⁺ (green) and HNA⁺ (red) neurons migrating in a chain in the hippocampal alvius. *f*, βIII-tubulin⁺ and MAP2⁺ (inset) neurons in the striatum, adjacent to the rostral migratory stream (green, bIII-tubulin and MAP2; red, HNA; yellow, double-stained human nuclei). *g*, Hu⁺ (red) HNA⁺ (green) neuron in the septum. *h*, GAD67⁺ (red) HNA⁺ (green) striatal neuron. Insets (*a–f*) show orthogonal projections of a high-power confocal image of each identified cell (arrow). Scale bars, 40 μm (*a–e*) or 20 μm (*f–h*).

b). In addition, abundant populations of HNA⁺ βIII-tubulin⁺ neurons were found in the olfactory subependyma and rostral migratory stream as well as in the hippocampal alvius (Fig. 6*e*). WMPC-derived neurons were also observed in the neostriatum, indicating striatal neuronal differentiation on the part of some xenografted WMPCs (Fig. 6*f*). These data showed that engrafted adult human WMPCs could integrate into the forebrain subventricular zone as neuronal progenitor cells that then gave rise to both granule and striatal neurons. Human WMPC-derived GFAP⁺ astrocytes and CNP⁺ oligodendrocytes were also common in recipient brains and were found primarily along the ventricles or in the subcortical white matter (Fig. 6*c* and *d*). Thus, adult human WMPCs showed context-dependent differentiation after xenograft to the developing rat brain and were competent to do so upon acute isolation, without the benefit of humoral instruction *in vitro*.

Discussion

These observations suggest that the WMPCs of the adult human forebrain include multipotential progenitor cells, capable of a finite and limited degree of expansion and self-renewal. These cells remain competent to respond to local instructive cues, with a wide range of lineage choices, upon xenograft as well as *in vitro*. They are readily able to give rise to neurons and glia once they are removed from their native white-matter environment. The freshly isolated adult WMPCs in our study did not require prolonged expansion to undergo neurogenesis *in vitro*, and seemed immediately competent to generate neurons upon xenograft to the developing brain.

Previous studies of the adult rat brain have identified parenchymal progenitor cells that are able to give rise to neurons and glia after a number of cell doublings, in the presence of bFGF⁹. In addition, nominally committed glial progenitor cells derived from the neonatal rat optic nerve have also been reported to give rise to neurons and oligodendrocytes⁴. The lineage diversification of these cells seems to require a humorally directed reprogramming of their phenotype, with the induction of an astrocytic intermediary on the way to neurogenesis. In the present study, adult human WMPCs did not seem to require any such reprogramming or transdifferentiation to achieve multilineage competence. Similarly, they did not seem to pass through an intermediate astrocytic stage before generating neurons, oligodendrocytes and astrocytes. Indeed, after their acute isolation and xenograft, A2B5-defined WMPCs were able to

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generate all major neural phenotypes *in vivo* and *in vitro*, without any exogenous growth factor exposure. Nevertheless, because an average of 7% of A2B5-sorted white-matter cells co-expressed GFAP (data not shown), it is possible that some WMPCs exhibit astroglial features at some point during their ontogeny, much like subventricular neural progenitor cells[26,27]. This categorization notwithstanding, our results suggest that the WMPCs of the adult human brain are fundamentally tissue-specific progenitor cells that are tonically restricted to glial lineage by the local parenchymal environment, and do not require specific phenotypic reprogramming for neuronal differentiation.

These data suggest that adult human WMPCs constitute a population of parenchymal glial progenitor cells whose *in situ* fate is restricted by the local white-matter environment. Yet the progenitor cell pool of the adult white matter may be heterogeneous, and it is not clear whether all WMPCs have the same ontogeny or fate potential[28-30]. A minority of multipotential progenitor cells might still persist among a larger pool of more fundamentally lineage-restricted glial progenitors[6]. These parenchymal multipotent progenitors may constitute a relatively rare subpopulation, more akin to persistent stem cells than to any lineage-restricted derivatives[31,32]. In this regard, although we did not detect telomerase activity in sorted WMPCs, if the clonogenic portion of these represents only a small fraction of the total progenitor pool, then their numbers might have been below the detection threshold of our TRAP assay. Further study of the heterogeneity of the white-matter progenitor cell population, and of the lineage competence of its constituent phenotypes, will be needed to define the spectrum of progenitor cell types in the adult brain. These considerations aside, multipotential and neurogenic progenitors are abundant in the adult human white matter and are both extractable and expandable. These cells may prove to be important agents for both induction and implantation strategies of cell-based neurological therapy.

Methods

Tissue dissociation and culture. Adult subcortical white matter was surgically obtained from 21 patients, including 14 undergoing epileptic resections (age 1–50 years; 7 males and 7 females), one undergoing aneurysmal repair (69-year-old male), 2 undergoing resections of a noncontiguous dysplastic focus (20-year-old male and 36-year-old female) and 4 undergoing traumatic temporal lobe decompressions (17–67 years old; all males). Samples were obtained from patients who consented to tissue use under protocols approved by the New York Hospital–Cornell and Columbia Presbyterian Hospital Institutional Review Boards. The samples were dissected and dissociated to single-cell suspensions using papain and DNase as described[2,33,34]. The cells were then suspended in DMEM/F12/N1 with either bFGF (20 ng/ml; Sigma, St. Louis, Missouri) alone or bFGF with NT-3 (2 ng/ml; R&D Minneapolis, Minnesota) and PDGF-AA (20 ng/ml; Sigma), and plated in 100-mm suspension culture dishes (Corning, New York).

Magnetic separation of A2B5+ cells. The number of viable cells was determined using calcein (Molecular Probes, Eugene, Oregon) 24–48 h after dissociation. The cells were then washed and incubated with A2B5 supernatant (clone 105; American Type Culture Collection, Manassas, Virginia) for 30–45 min at 4 °C, washed 3 times with PBS containing 0.5% BSA and 2 mM EDTA, and incubated with microbead-tagged mouse-specific rat IgM (1:4; Miltenyi Biotech, Bergisch Gladbach, Germany) for 30 min at 4 °C. The A2B5+ cells were washed, resuspended and separated using positive selection columns, type MS+ RS+ or LS+ VS+ (magnetic cell sorting (MACS); Miltenyi Biotech). For flow cytometry of matched samples, cells were incubated in FITC-labeled mouse-specific goat IgM at 1:50 before FACS.

Transfection and sorting. Samples were transfected with pCNP2:hGFP after 2–6 d *in vitro*, using 2 µg of plasmid DNA and 10 µl of Lipofectin

(Gibco, Carlsbad, California) as described[2,33,35]. Sorting for pCNP2:hGFP and A2B5 immunofluorescence was performed on a Becton-Dickinson FACS Vantage (San Diego, California), also as described[2,33,35]. Untransfected and IgM-exposed control cells were used to calibrate background; a false-positive rate of 1% was accepted as cutoff.

Generation of primary and secondary spheres. A2B5+ and A2B5-depleted white-matter cells were distributed to a 24-well plate directly after sorting, at 100,000, 50,000, 25,000, 10,000, 5,000 and 1,000 cells/ml with 0.5 ml/well of DMEM/F12/N1 with bFGF/NT3/PDGF-AA. The resulting WMPC-derived neurospheres were passaged at the 50- to 100-cell stage, by dissociation to single cells with trypsin and EDTA. The cells were plated at 3,000 cells/well. Three weeks later, the resultant secondary spheres were either dissociated and passaged again as tertiary spheres, or plated into 2% FBS with 20 ng/ml brain-derived neurotrophic factor on a polyornithine and fibronectin substrate and fixed 2 weeks later.

Lentiviral tagging and lineage analysis. A2B5-sorted cells were infected 2–5 d after separation with lentivirus (10⁸ PFU/ml) expressing GFP under CMV promoter control and a WPRE5 post-transcriptional regulatory element[12,13]. The lentivirus was generated by co-transfecting plasmids pCMV/DR8.91, pMD.G, and pHRCMVGFPwsin into 293T cells as described[14]. A2B5-sorted cells were exposed to lentivirus for 24 h in polybrene-supplemented medium (8 µg/ml), then passaged into fresh medium in 24-well plates. GFP expression by tagged cells was observed within 2 d. The primary spheres that arose in these cultures were dissociated 3 weeks later and replated at 3,000 cells/well; secondary spheres arose from these within 2 weeks.

TRAP assay. Telomerase activity was determined using the TRAP assay[23,24], described in detail in the material accompanying Supplementary Figure 1 online.

In utero transplantation. Transuterine xenograft into E17 rat fetuses was performed as described[21,36]. Some cells were injected within 24–48 h after sorting and others after 10 d *in vitro* in FGF2, PDGF-AA and NT3. One month after implantation, the animals were perfusion-fixed by 4% paraformaldehyde. Experiments were conducted with the approval of the Institutional Animal Care and Use Committee of the Weill Medical College of Cornell University.

Immunocytochemistry. Xenografted rat brains were cryosectioned at 15 µm, permeabilized with PBS, 0.1% saponin and 1% NGS, and blocked with PBS, 0.05% saponin and 5% NGS, each for 30 min. Sections were labeled with HNA-specific mouse antibody (1:50; Chemicon, Temecula, California), then immunostained with βIII-tubulin–specific antibody TuJ1 (1:600; Covance, Princeton, New Jersey), MAP2-specific antibody AP-20 (1:50; Sigma), HuC/HuD-specific mouse monoclonal antibody 16A11 (25 µg/ml; H. Furneaux, Memorial Sloan-Kettering Cancer Center, New York), GAD67-specific rabbit antibody (1:100; Chemicon), GFAP-specific mouse antibody SMI 21 (1:1,000; Sternberger, Lutherville, Maryland), GFAP-specific rabbit antibody (1:400; Sigma), CNP-specific mouse antibody SMI 91 (1:1000 Sternberger), human nestin–specific rabbit antibody (1:200; Chemicon), or doublecortin-specific rabbit antisera (1:100; C. Walsh, Harvard Medical School, Boston, Massachusetts). The sections were incubated with antibody overnight at 4 °C. Species- and isotype-specific fluorescent secondary antibodies were applied at 1:100 for 1.5 h at room temperature.

O4 and A2B5 were immunolabeled *in vitro* as described[2]. For multiple-antigen labeling, O4 was localized on live cells that were then fixed and stained for βIII-tubulin, MAP2, GFAP, Hu, GAD67 or BrdU. O4 supernatant (R. Bansal and S. Pfeiffer, University of Connecticut Health Center, Farmington, Connecticut) was used at 1:100 for 40 min at 4 °C. Antibodies against βIII-tubulin, MAP-2, GFAP and BrdU (BrdU-specific rat antibody; 1:200; Harlan, Indianapolis, Indiana) were incubated overnight at 4 °C. Fixed cultures were counterstained with DAPI (10 µg/ml; Molecular Probes).

Confocal imaging. In the xenografted brains, single cells that appeared co-labeled for both human- and cell-specific markers were evaluated by confocal imaging as described[21,37]. To be deemed double labeled, cells were

required to have HNA-specific signal surrounded by neuronal or glial immunoreactivity in every serially acquired 0.4-μm z-dimension optical section, as well as in each orthogonal side view thereof.

Calcium imaging. Outgrowths from both first- and second-passage WMPC-derived neurospheres were assessed 2–3 weeks after plating into BDNF-supplemented DMEM/F12/N1 with 2% FBS. These mixed neuronal and glial outgrowths were challenged with 100 μM glutamate or 60 mM potassium. Cytosolic calcium imaging was conducted using confocal microscopy of cultures loaded with Fluo-3 acetoxymethylester (Molecular Probes)[33,38,39]. We previously reported that adult progenitor–derived human neurons showed a mean calcium rise of >400% in response to 60 mM potassium in vitro, compared with glial responses of <20%[38]. In this study, we assigned neuronal identity to cells with ≥2-fold calcium elevations to depolarization.

Electrophysiology. Sister cultures to those subjected to calcium imaging were assessed by whole-cell patch-clamp analysis. Whole-cell voltage-clamped recordings of fiber-bearing cells were conducted and analyzed as described[15,33]. A holding potential of ~60 mV and voltage steps of 10 mV with 100-ms durations were applied to the recorded cells through the patch electrodes. Signals were sampled every 50 μs.

Note: Supplementary information is available on the Nature Medicine *website.*

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EVIDENCE APPENDIX
EXHIBIT 17

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Progenitor Cells of the Adult Human Subcortical White Matter

Neeta S. Roy, Martha S. Windrem, and Steven A. Goldman

OLIGODENDROCYTE PROGENITOR CELLS OF THE ADULT MAMMALIAN BRAIN

Neural Progenitor Cells of the Adult Brain

Over the past few decades, historic notions of the structural immutability and cellular constancy of the adult vertebrate brain have been largely dispelled. Neurogenesis was first demonstrated in the rodent olfactory bulb and the hippocampus (Altman and Das, 1965, 1966; Kaplan and Hinds, 1977; Kaplan, 1985) and the songbird vocal control centers (Goldman and Nottebohm, 1983; Nottebohm, 1985). The phenomenon of adult neurogenesis has now been described throughout vertebrate phylogeny (Goldman, 1998), including monkeys (Gould *et al.*, 1998) and humans (Eriksson *et al.*, 1998; Kirschenbaum *et al.*, 1994; Pincus *et al.*, 1998). In all species yet examined, newly generated neurons seem to be generated from multipotential stem cells, the principal source of which appears to be the periventricular subependyma (SVZ) (Goldman *et al.*, 1993; Lois and Alvarez-Buylla, 1993). In addition, restricted pools of mitotically competent but phenotypically biased neuronal progenitor cells appear to derive from these stem cell populations. These neuronally restricted pools include the anterior subventricular zone of the forebrain and its rostral extension through the olfactory subependyma, as well as the subgranular zone of the hippocampus, each of which give rise almost exclusively to neurons *in vivo*. However, persistent multipotential stem cells have been reported in cultures derived from each of these regions (Gage *et al.*, 1998), suggesting that the apparent neuronal restriction of these progenitor populations may reflect not the inherent lineage capacity of the cells, so much as local environmental signals biasing toward neuronal differentiation (Seaberg and van der Kooy, 2002).

Besides these persistent neuronal progenitors and multipotential neural stem cells, more restricted lineages of glial progenitor cells also persist in the adult brain, in both the residual ventricular zone (Levison and Goldman, 1993; Luskin, 1993), as well as dispersed throughout the subcortical and cortical parenchyma (Gensert and Goldman, 1996; Levine *et al.*, 2001; Noble, 199 ; Reynolds and Hardy, 1997). Indeed, in contrast to the restricted distribution of neuronal progenitor cells and SVZ stem cells, oligodendrocyte progenitor cells (OPCs) seem to be extraordinarily widespread in the adult mammalian brain.

Oligodendrocyte Progenitors of the Normal Adult Rodent Brain

The principal class of OPCs in adult rodents is a bipotential astrocyte-oligodendrocyte progenitor cell designated the O-2A progenitor, by virtue of its generation *in vitro* of oligodendrocytes and type 2 astrocytes, the latter comprising the traditionally recognized fibrous astrocytes of the white matter. These cells were initially isolated from the optic nerves of perinatal rats, as O-2A progenitors (Raff *et al.*, 1983b). In neonatal rats, OPCs are characterized by expression of the GD3 and GQ gangliosides, the latter recognized by the monoclonal antibody A2B5, which has been used to identify this cell population (Noble *et al.*, 1992). Though similar progenitors were long ago reported in the adult optic nerve (Vaughn, 1969), the isolation of adult OPCs, or O-2A^{Adult} progenitors, was only accomplished relatively recently in rodents (Ffrench-Constant and Raff, 1986; Wolswijk and Noble, 1989). These cells have since been isolated from the adult rat ventricular zone, spinal cord, cerebellum, and subcortical white matter (Engel and Wolswijk, 1996; Gensert and Goldman, 1996; Levine *et al.*, 1993).

Antigenic Recognition of Adult OPCs

Little is known about the natural history of the adult OPC in normal adults. In histological sections of the adult rodent brain, OPCs have mainly been identified by their expression of both NG2 chondroitin sulfate proteoglycan (Levine *et al.*, 1993; Nishiyama *et al.*, 1997) and the platelet derived growth factor-alpha receptor (PDGF-αR). The expression of PDGF-αR and the NG2 epitope substantially overlaps in rats (Nishiyama *et al.*, 1996; Pringle *et al.*, 1992). Moreover, a persistent population of O4/NG2 co-expressing cells has been demonstrated in the adult rat cerebral cortex, effectively bridging the antigenic gap between early and committed OPCs (Reynolds and Hardy, 1997). On the basis of these studies, NG2-immunoreactivity has been developed as a surrogate marker for parenchymal oligodendrocyte progenitor cells. In addition, adult-derived OPCs have several features that may allow them to be distinguished: Whereas the perinatal OPC utilizes vimentin as an intemediate filament and does not express the oligodendrocytic sulfatide recognized by Mab O4, its adult counterpart does not express vimentin, but does express O4 (Shi *et al.*, 1998; Wolswijk and Noble, 1989; Wolswijk *et al.*, 1991). These parenchymal OPCs are present in both gray and white matter, and exist *in vivo* as extensively branched cells. The NG2 population represents as many as 5–8% of all the cells in the adult rodent brain (Dawson *et al.*, 2000); this is congruent with earlier estimates that 5% of all glia in the optic nerve may be progenitors (Vaughn and Peters, 1968).

Turnover

OPCs in the adult brain may include both slowly dividing cells in normal parenchyma and a quiescent cell population that responds only to injury or demyelination. *In vivo* studies of the adult cerebellar cortex reveal the presence of slowly dividing OPCs with a mitotic index of 0.2 to 0.3% (Levine *et al.*, 1993). Nevertheless, OPCs seem to constitute the main cycling population of the adult brain parenchyma. Bromodeoxyuridine (BrdU) labeling of the intact spinal cords of 13- to 14-week-old rats has shown that 10% of all cells in the white matter incorporated BrdU, of which 70% expressed NG2. In animals maintained for 4 weeks after BrdU injection, BrdU-labeled astrocytes and oligodendrocytes were noted, indicating that the cycling NG2 cells would have generated both cell types (Horner *et al.*, 2000). In studies using retroviral labeling to mark dividing cells, 35% of the cycling cells in the adult cortex co-labeled with NG2, and these were distinctly present as clusters. Furthermore, these NG2-positive clusters doubled in size every 3 months (Levison *et al.*, 1999). Using similar retroviral labeling techniques, the presence of cycling cells that preferentially give rise to oligodendrocytes has been shown in both the subventricular zone (SVZ) and subcortical white matter of adult rats (Gensert and Goldman, 1996; Levison and Goldman, 1993).

Lineage Potential

Previous studies had concluded that the perinatal OPC has a limited life span *in vivo*, which was attributed to a pattern of "exhaustive" symmetrical division and differentiation in

oligodendrocytes (Temple and Raff, 1986). Yet OPCs now appear to be maintained throughout life. This suggests that at least a fraction of OPCs may arise through a self-renewing, asymmetrical divisions, such that OPCs generate both differentiated progeny and themselves (Wren et al., 1992). Indeed, adult OPCs of both rodents and humans retain their ability to generate oligodendrocytes and astrocytes over several generations in vitro (Tang et al., 2000).

It seems likely that perinatal OPCs are the source of adult OPCs. Using time-lapse microcinematography, it has been shown that "founder cells" exhibiting properties of perinatal OPCs eventually give rise to cells with the properties of adult OPCs (Wren et al., 1992). As noted, just as repetitive passage of perinatal OPCs gives rise to cells with adult OPC-like properties (Wolswijk et al., 1990), slowly dividing adult OPCs can respond to FGF and PDGF by assuming the more rapid expansion kinetics typical of perinatal OPCs. Together, these data argue that perinatal and adult OPCs constitute two points along the differentiation spectrum of a common lineage. Nonetheless, diversification within that lineage may nonetheless have resulted in substantial phenotypic heterogeneity among adult OPCs (Gensert and Goldman, 2001).

Humoral Control of Oligoneogenesis

Adult and perinatal OPCs share many commonalities in their responses to humoral growth factors, but nonetheless exhibit differential responses to both neural mitogens and differentiation agents. These include, but are by no means limited to, the following:

1. Platelet derived growth factor. PDGF is perhaps the most prominent described oligotrophin and has been implicated in both the mitotic expansion of OPCs and their initiation of terminal lineage commitment (Hart et al., 1989a; Noble et al., 1988; Raff et al., 1988; Wolswijk et al., 1991). OPCs uniquely express high levels of PDGFα receptor, and can be specifically identified on that basis (Ellison and de Vellis, 1994; Fruttiger et al., 1999; Hart et al., 1989b). In response to PDGF, both perinatal and adult OPCs enter the mitotic cycle. However, cycling time differs in the two cell populations, in that adult OPCs have a slow, 3- to 4-day cell cycle, whereas perinatal OPCs divide daily (Noble et al., 1988); (Wolswijk et al., 1991). In OPCs derived from the adult spinal cord, PDGF alone supports the slow mitotic expansion of OPCs, as the cells divide slowly and undergo asymmetrical division, generating a differentiated oligodendrocyte and another progenitor (Engel and Wolswijk, 1996). However, in the presence of PDGF and FGF, adult OPCs accelerate their cycle progression, dividing rapidly and apparently symmetrically to yield additional progenitors. They then assume the bipolar morphology and A2B5 immunoreactivity of oligodendrocyte progenitor cells, but fail to generate oligodendrocytes without downstream inductive differentiation. As a corollary to this "perinatalization" of adult-derived OPCs, cultures of perinatal OPCs expanded over long periods of time in the presence of PDGF alone develop the cyclicity of adult OPCs (Tang et al., 2000). These results suggest that in rodents at least, perinatal and adult-derived OPCs represent points on a continuum of differentiative state, rather than discrete phenotypes

2. Fibroblast growth factor. FGF differentially regulates OPC proliferation and differentiation in culture and modulates gene expression of its own receptors in a developmental and receptor type-specific manner (Bansal et al., 1996). Most in vitro studies show that bFGF is a major mitogen for cells in the oligodendrocyte lineage (Besnard et al., 1989; Eisenbarth et al., 1979). It has been shown to stimulate the proliferation of late progenitors and inhibit their terminal differentiation (Bansal and Pfeiffer, 1994; McKinnon et al., 1990). More important, it establishes the responsiveness to PDGF by up-regulating the expression of PDGF-αR (McKinnon et al., 1990). Most studies with adult OPCs show that bFGF is most mitogenic when used in combination with PDGF (Mason and Goldman, 2002; Tang et al., 2000). Recently it has been shown that OPCs maintained in the presence of bFGF eventually become resistant to replicative senescence (Tang et al., 2001). Besides its well-documented effect on OPCs, bFGF also induces the down-regulation of myelin genes, such as myelin basic protein (MBP), in mature oligodendrocytes without reverting

them to the progenitor phenotype or effecting reentry into the cell cycle (Bansal and Pfeiffer, 1997; Grinspan et al., 1993).

3. *Neurotrophin-3 (NT3)*. Whether NT3 has proliferative or differentiative effect on OPCs is yet unresolved. One study indicated that NT3, specifically in combination with PDGF, is proliferative for post-natal OPCs both *in vitro* and *in vivo* (Barres et al., 1994b). Other studies, however, found that NT3 is not proliferative for adult OPCs alone or in combination with PDGF and bFGF (Engel and Wolswijk, 1996; Ibarrola et al., 1996). Perhaps this differential response may be a function of the different OPC-types that have been used for the two studies. In the contused adult spinal cord, NT3 has been shown to increase OPC proliferation and myelination (McTigue et al., 1998). A recent *in vitro* study with OPCs from adult spinal cord dissociates indicates that NT3 induced myelination and the proliferation of O4+/O1-cells (Yan and Wood, 2000).

4. *Neuregulin*. The neuregulins are a family of soluble and transmembrane protein isoforms, of which glial growth factor 2 (GGF2) is a member (Adlkofer, 2000). The neuregulins act upon erbB receptors, in particular on the erbB2, 3, and 4 heterodimeric receptors (Buonanno and Fischbach, 2001). Perinatal OPCs divide in response to GGF provided cAMP levels are high, so that adenyl cyclase and erbB stimulation may operate synergistically as glial progenitor mitogens (Shi et al., 1998). Canoll et al. observed a similar proliferative effect on $O4^+/O1^-$ progenitors (Canoll et al., 1996). Adult OPCs respond to GGF2 as well, although their mitogenic activation by GGF2 appears to require the concurrent activation of the PDGF receptor, along with elevated cAMP. An interesting feature of neuregulins includes their induction of phenotypic reversion by differentiated oligodendrocytes (Canoll et al., 1999). OPCs produce neuregulins (Raabe et al., 1997) as well as respond to it (Shi et al., 1998). Since they express full-length neuregulin erbB receptors, OPCs may utilize neuregulins as an autocrine factor, as well as a neuronally derived oligotrophin (Fernandez et al., 2000). This is likely to obtain in the environment of the adult human white matter, from which oligodendrocytes have similarly been shown to produce neuregulins and express receptors to them (Cannella et al., 1999; Deadwyler et al., 2000).

5. *Triiodothyronine*. When OPCs derived from optic nerves or cerebral hemispheres are cultured in the presence of T3, they immediately stop dividing and differentiate into oligodendrocytes. In fact, the number of times an OPC can divide varies inversely with its concentration of T3, implicating T3 as an oligodendrocytic differentiation factor (Baas et al., 1997). T3 seems to play a major role in controlling the timing of OPC differentiation (Barres et al., 1994a). Accordingly, hypothyroid states have been associated with deficits in early myelination in neonatal cretinism, which may reflect a failure in T3-mediated OPC expansion.

6. *Insulin growth factor-1 (IGF-1)*. During development, high levels of IGF1 are observed just before active myelination commences (Bach et al., 1991; Carson et al., 1993). IGF-1 increases proliferation and survival, enhance differentiation, and modulate the expression of MBP in both OPCs and oligodendrocytes (Barres et al., 1992; McMorris and Dubois-Dalcq, 1988; Saneto et al., 1988).

Oligodendrocyte Progenitors of the Adult Human Brain

The earliest evidence that the adult human brain harbors oligodendrocyte progenitors came from early studies of MS lesions. Histopathologically, these lesions were found to harbor regions of extensively remyelinated axons, as well as numerous free oligodendrocytes (Moore et al., 1985; Prineas and Connell, 1979; Prineas et al., 1984). Subsequent studies identified populations of immature cells expressing the neural carbohydrate epitope HNK1; these were postulated to comprise early oligodendroglia, although these early studies were unable to identify any definitive oligodendrocyte progenitor cell phenotype (Prineas et al., 1989; Wu and Raine, 1992).

PDGF-αR expressing OPCs have been shown in both MS lesions and surrounding normal white matter (Scolding et al., 1998). These PDGF-αR$^+$ cells were found to be more

frequent in or near MS lesions compared to normal surrounding white matter (WM), and those near lesions were more often cycling, as revealed by immunoreactivity for Ki67, a marker of proliferation (Maeda et al., 2001). Corroborating these observations with another marker of phenotype, the NG2 chondroitin sulfate proteoglycan was demonstrated in both normal adult human WM and MS lesions. As in their rodent counterparts, human NG2$^+$ cells were found to be extensively ramified. Cells morphologically similar to NG2$^+$ cells were reported to express PDGF-αR as well, although co-expression of the two by a common phenotype has yet to be directly demonstrated.

Premyelinating oligodendrocytes—defined by their expression of proteolipid protein (PLP), and their contiguity with axons despite an absence of attendant ensheathment— have also been shown in such MS lesions (Chang et al., 2002). Interestingly, NG2$^+$ cells are virtually absent from lesions lacking premyelinating oligodendrocytes. This suggests that NG2$^+$ cells might be the source of these premyelinating oligodendrocytes. However, the NG2 chondroitin sulfate may not be specific to OPCs in the adult human brain, as microglial cells express or sequester high levels of NG2-IR (Pouly et al., 1999; also Nunes, Roy, and Goldman, unpublished observations). Indeed, in dissociates of both fetal and adult human brain tissue, most NG2$^+$ cells were microglial (Pouly et al., 1999). To establish a more reliable marker of OPCs in adult human tissues, Scolding et al. thus assessed the phenotypic specificity of two cardinal markers of OPC phenotype in rodents, specifically the PDGF-α receptor and the A2B5 epitope represented by the GQ ganglioside. By scoring the incidence of both PDGF-αR$^+$ and A2B5$^+$ cells in tissue print preparations of adult human white matter, Scolding and colleagues determined that these markers recognize a common parenchymal progenitor cell population. On this basis, they were able to report the first estimates of the incidence of oligodendrocyte progenitor cells in the human white matter (Scolding et al., 1999).

Despite this wealth of histological assessment of parenchymal progenitor cells, relatively few studies have yet correlated the antigenic expression patterns of single parenchymal phenotypes with their lineage potential, either in vivo or in vitro. As a result, it remains unclear if the expression of markers such as GD3, NG2, A2B5, or PDGF-αR is specific to adult OPCs, or whether it instead is shared among different, already discrete lineages at similarly early points in their phenotypic specification. The uncertain lineage potential of histologically antigen-defined oligodendrocyte progenitor cells has derived in part from an historic inability to identify or isolate these cells from human brain tissues. An early attempt to identify oligodendrocyte progenitors in dissociates of adult human brain (Kim et al., 1983) was followed by successful in vitro and in vivo demonstrations of immature oligodendroglia, which were termed pro-oligodendrocytes because of their post-mitotic state. These cells were defined as being O4$^+$/A2B5$^-$/GalC$^-$ (Armstrong et al., 1992). Pro-oligodendrocytes were further characterized and found to express the PDGF-αR in tissue, where they were estimated to constitute 2% of the total cell population (Gogate et al., 1994). Subsequent studies of the adult human white matter in vitro revealed the presence of mitotic cells that could give rise to oligodendrocytes, though the identity of the precursor remained unclear (Roy et al., 1999; Scolding et al., 1995);.

Humoral Control of Adult Human Oligodendrocyte Progenitor Cells

Human and rodent OPCs differ not only in their antigenic expression patterns, as noted, but also as in their responses to humoral growth factors. Adult human OPCs do not proliferate in response to bFGF, PDGF, or IGF-1, each of which can act singly as a mitogen for rodent OPCs (Armstrong et al., 1992; Gogate et al., 1994; Prabhakar et al., 1995). Instead, in human OPCs, IGF-1 has been shown to increase the proportion of post-mitotic pro-oligodendrocytes and to promote the maturation of these cells as oligodendrocytes (Armstrong et al., 1992). Human OPCs also seem to be mitotically unresponsive to astrocyte conditioned medium (Armstrong et al., 1992; Gogate et al., 1994; Prabhakar et al., 1995; Scolding et al., 1995). As noted previously, neuregulin supports the expansion of OPCs and is released by neurons in an activity-dependent manner that might allow the activity-dependent modulation of OPC expansion (Canoll et al., 1996). However, these

observations have yet to be verified as operative in human OPCs. Indeed, little data are available on the factor responsiveness of human OPCs, despite the overt clinical importance of establishing the optimal expansion and differentiation conditions for these cells. Rather, the study of their growth factor responsiveness, patterns of receptor expression, and likely paracrine interactions with other parenchymal cell populations have been impeded by the inability to identify and isolate OPCs from the adult human brain, and hence the lack of material for molecular and cellular analysis.

Isolation of Adult Human Oligodendrocyte Progenitor Cells

To address the need for isolating enriched populations of adult OPCs, we used promoter-specified fluorescent activated cell sorting (FACS) to identify and extract these cells from adult human brain tissue. Traditionally, FACS has been used to sort live cells on the basis of surface antigen expression, particularly in the hematopoetic system, in which FACS has been used to define and isolate the major stem cell and intermediate progenitor phenotypes generated during lymphopoesis and hematopoiesis. However, the application of FACS to the nervous system was stymied by the lack of identified surface antigens specific to stage or phenotype among neural cells. Yet in 1994, the green fluorescence protein was first identified as a live cell reporter of gene expression (Chalfie et al., 1994). By placing GFP under the transcriptional control of promoters regulating the expression of cell-specific genes, we were able target specific cell phenotypes for FACS isolation. We first applied this approach to extracting neuronal progenitor cells from the fetal ventricular zone (Wang et al., 1998), by transducing ventricular zone cells with GFP placed under the control of the Tα1 tubulin promoter, an early neuronal regulatory sequence (Gloster et al., 1994; Miller et al., 1987, 1989). This approach has since allowed us to isolate neuronal progenitor cells from both the adult human ventricular zone (VZ) and hippocampus (Roy et al., 2000a, 2000b). In addition, by modifying our choice of promoters to those specifically active in even earlier neural progenitors, we were able to isolate less committed neural stem cells from both the adult and fetal human brain (Keyoung et al., 2001; Roy et al., 2000a, 2000b).

The development of promoter-based FACS gave use the means to identify and then isolate oligodendrocyte progenitor cells from the adult human brain (Fig. 10.1). To this end, we used the early promoter for an early oligodendrocyte protein, 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNP) (Scherer et al., 1994; Vogel and Thompson, 1988). CNP protein is the earliest myelin-associated protein known to be expressed in developing oligodendrocytes. It is expressed by oligodendrocytes at all ontogenetic stages (Sprinkle, 1989; Weissbarth et al., 1981), including by newly generated cells of oligodendrocytic lineage within the subventricular zone and their mitotic precursors (Scherer et al., 1994; Yu et al., 1994). The 5' regulatory region of the CNP gene includes two distinct promoters, P2 and P1, which encode for two CNP isoforms, CNP1 (46kDA) and CNP2 (48 kDa). These promoters are sequentially activated during development, with the more upstream P2 promoter (P/CNP2) directing transcription to immature oligodendrocytes and their progenitors (Gravel et al., 1998; O'Neill et al., 1997). On this basis, P/CNP2 was chosen to identify oligodendrocyte progenitors from adult human subcortical white matter (Roy et al., 1999). P/CNP2:hGFP was transfected into dissociate of adult human white matter, and following GFP expression 3 to 4 days later, the P/CNP2:GFP$^+$ cells were isolated by FACS (Roy et al., 1999). These cells, maintained in serum-deficient media supplemented with FGF2, PDGF, and NT-3, were bipolar, immunoreactive for A2B5, incorporated BrdU from their culture media, and developed into O4$^+$ oligodendrocytic in vitro (Fig. 10.2). These data indicated that the P/CNP2:hGFP-defined cells were mitotic oligo-dendrocyte progenitors. On this basis, P/CNP2:hGFP$^+$ oligodendrocyte progenitors were extracted directly from adult human WM dissociates using FACS. We found that an average of 0.5 $\pm$ 0.1% of all white matter cells directed P/CNP2:hGFP expression. Given a transfection efficiency of 13.5%, determined using the percentage of GFP expressing cells obtained with p/CMV:GFP for noncell type specific transfection, it could be estimated that over 4% of adult human subcortical WM are P/CNP2-defined progenitors. Immediately after FACS, these P/CNP2:hGFP-separated cells were initially bipolar, and
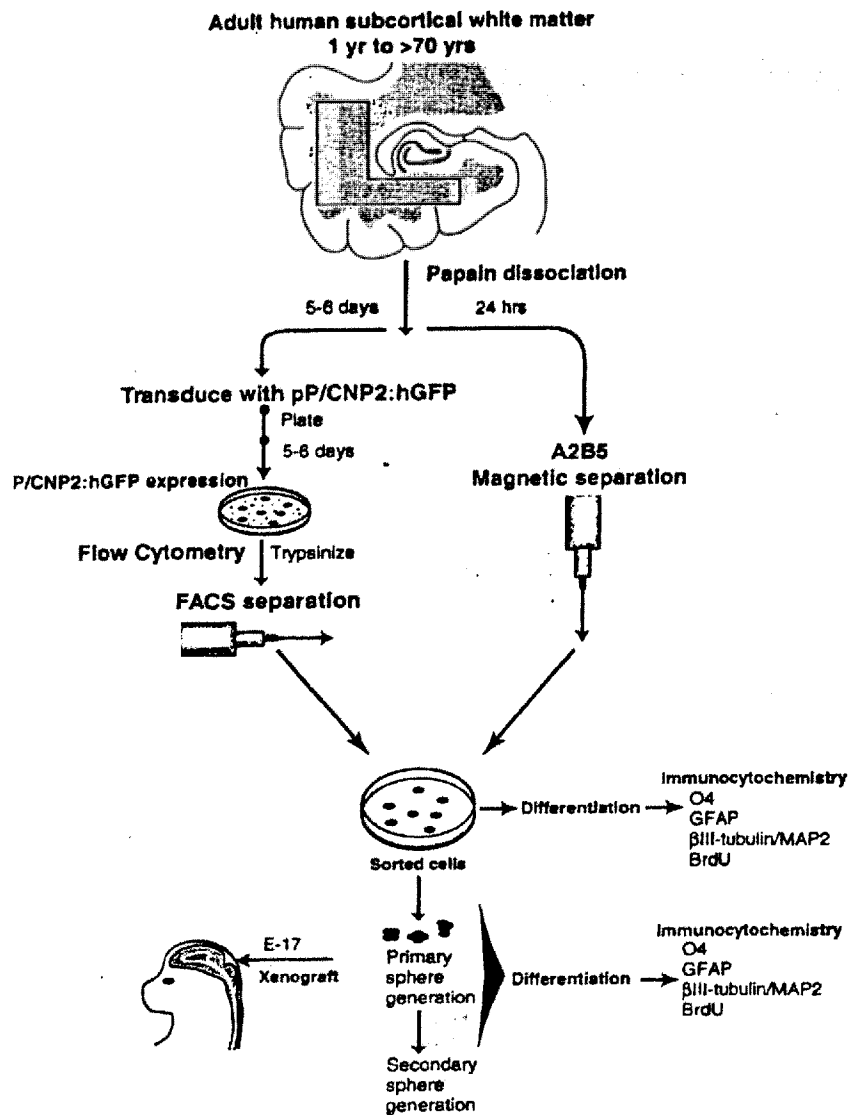
Adult human subcortical white matter
1 yr to >70 yrs

Papain dissociation

5-6 days   24 hrs

Transduce with pP/CNP2:hGFP
Plate
5-6 days
P/CNP2:hGFP expression

A2B5
Magnetic separation

Flow Cytometry | Trypsinize

FACS separation

Differentiation →
Immunocytochemistry
O4
GFAP
βIII-tubulin/MAP2
BrdU

Sorted cells

E-17
Xenograft

Primary
sphere
generation

Differentiation →
Immunocytochemistry
O4
GFAP
βIII-tubulin/MAP2
BrdU

Secondary
sphere
generation

FIGURE 10.1

Oligodendrocyte progenitor cells may be specifically targeted and isolated from the white matter. This schematic outlines basic strategies for isolating oligodendrocyte progenitor cells from the adult white matter, using either fluorescence-activated cell sorting (FACS) or a higher-yield, less specific alternative immunomagnetic isolation (MACS).

expressed the early oligodendrocytic marker A2B5, but none of the differentiated markers O4, O1, or galactocerebroside; over half incorporated BrdU. When followed up to a month in culture, >80% of the PCNP2:hGFP$^+$ cells become oligodendrocytes, progressing through a succession of A2B5, O4, and galactocerebroside expression, recapitulating the developmental sequence of antigenic expression (Noble, 1997). Thus, with this strategy not only was the existence of oligodendrocyte progenitors established in adult human white matter, but a method was developed to separate the progenitors in a form appropriate for engraftment and further analysis.
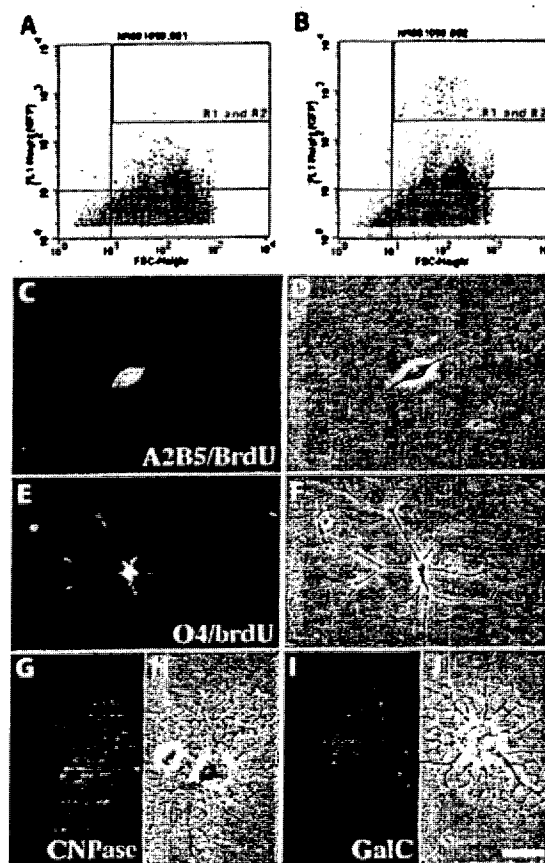
FIGURE 10.2

Sorted human white matter progenitor cells typically mature as oligodendrocytes. (A–B) A representative sort of a human white matter sample, derived from the frontal lobe of a 42-year-old woman during repair of an intracranial aneurysm. This plot shows 50,000 cells (sorting events) with their GFP fluorescence intensity plotted against forward scatter (a measure of cell size). Part A indicates the sort obtained from a nonfluorescent P/hCNP2:lacZ-transfected control, while part B indicates the corresponding result from a matched culture transfected with P/hCNP2:hGFP. (C–D) A bipolar A2B5⁺/BrdU⁺ cell, 48 hours after FACS. (E–F) By 3 weeks post-FACS, P/CNP2:hGFP-sorted cells developed multipolar morphologies and expressed oligodendrocytic O4 (red). These cells often incorporated BrdU, indicating their in vitro origin from replicating A2B5⁺ cells. (G–I) Matched phase (G, I) and immunofluorescent (H, J) images of maturing oligodendrocytes, 4 weeks after P/CNP2:hGFP-based FACS. These cells expressed both CNP protein (H) and galactocerebroside (J), indicating their maturation as oligodendrocytes. Scale bar = 20 μm. Taken from Roy et al., 1999; with permission.

Antigenicity of Oligodendrocyte Precursor Cells

As described earlier, virtually all P/CNP2:hGP-defined OPCs are immunoreactive for A2B5 (Roy et al., 1999). This permitted us to use A2B5-based sorting to increase the yield of isolated progenitors, to numbers sufficient for experimental transplantation. Although both immature neurons and glia express A2B5-immunoreactivity during development (Aloisi et al., 1992; Eisenbarth et al., 1979; Lee et al., 1992), the adult subcortical parenchyma is relatively devoid of young neurons, allowing A2B5 to be used as a selective marker of glial and oligodendrocyte progenitor cells (Raff et al., 1983a; Satoh et al., 1996; Scolding et al., 1999). The specific use of A2B5 as an antigenic surrogate for P/CNP2:hGFP-defined OPCs has thus constituted a significant practical advance. By extracting OPCs via A2B5-based surface-antigen based sorting, the limitations of transfection-based tagging, which include

direct cytotoxicity as well as low efficiency, can be avoided entirely. As a result, the practical issue of acquiring sufficient numbers of viable OPCs to permit transcriptional and biochemical analysis, as well as engraftment studies, can now be effectively addressed.

Multipotential Progenitors of the Adult Human White Matter

Like their lower species counterparts, human OPCs may not be strictly dedicated or autonomously programmed to oligodendrocytic differentiation. When purified from adult human subcortical tissue, derived from surgically resected temporal lobe, white matter progenitor cells (WMPCs) give rise largely to oligodendrocytes. However, when grown under conditions of very low density, we noted that these cells also generate occasional neurons (Roy et al., 1999). On this basis, we asked whether the white matter progenitor cells of the adult human brain might actually constitute a type of multipotential neural progenitor or neural stem cell. We found that white matter progenitor cells, purified by FACS from the adult human brain, can indeed generate neurons as well as both major glial cell types—astrocytes and oligodendrocytes—when raised in culture under conditions of high purity and low density (Nunes et al., 2003). Under these conditions, the cells are effectively removed from other cells, as well as from the proteins that other cells may secrete. Under these conditions, the sorted progenitor cells divide and expand as multipotential clones that generate neurons as readily as oligodendrocytes (Fig. 10.3). They can continue to divide and expand for several months in culture, dividing to increase their
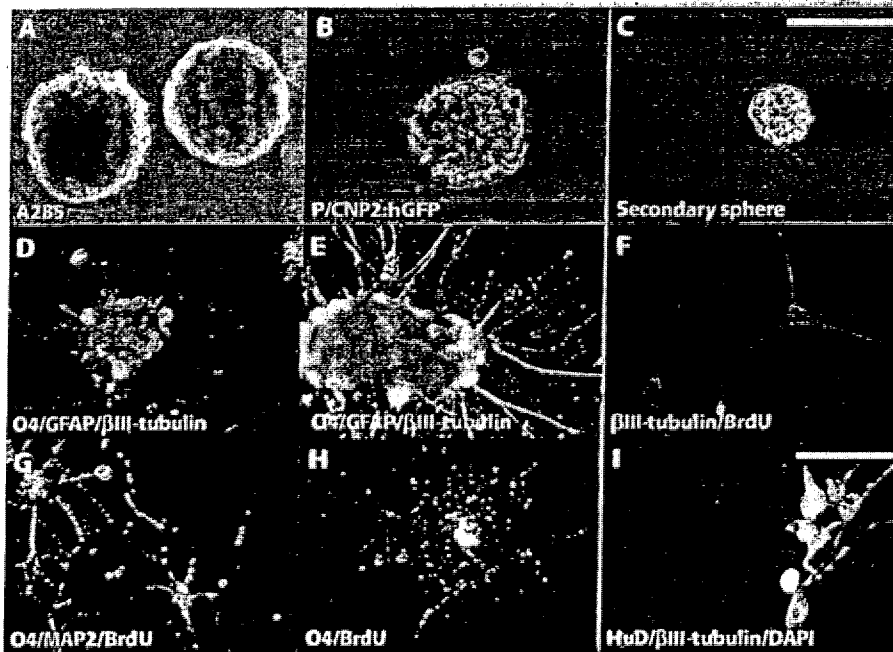

FIGURE 10.3

Adult human WMPCs give rise to multipotential neurospheres. (A) First-passage spheres generated from A2B5-sorted cells 2 weeks post-sort. (B) First-passage spheres arising from P/CNP2:hGFP sorted cells, 2 weeks. (C) Second-passage sphere derived from an A2B5-sorted sample, at 3 weeks. (D) Once plated onto substrate, the primary spheres differentiated as βIII-tubulin⁺ neurons (red), GFAP⁺ astrocytes (blue), and O4⁺ oligodendrocytes (green). (E) Neurons (red), astrocytes (blue), and oligodendrocytes (green) similarly arose from spheres derived from P/CNP2:GFP-sorted WMPCs. (F–H) BrdU incorporation (blue) revealed that new neurons (F: βIII-tubulin in red; G: MAP2 in red) and oligodendrocytes (H: O4 in green) were both generated in vitro. (I) βIII-tubulin⁺ neurons (green) co-expressed neuronal Hu protein (Barami et al., 1995; Marusich et al., 1994) (red, yielding: yellow double-label). Nuclei counterstained with DAPI (blue). From Nunes et al. (2003). Scale: A–E, 100 µm; F–I, 24 µm.

numbers in the process. Moreover, upon xenograft to the developing fetal rat forebrain, adult human WMPCs can mature into neurons as well as oligodendrocytes and astrocytes *in vivo*, in a region- and context-dependent manner (Fig. 10.4). The nominally glial progenitor cell of the adult human white matter thus appears to constitute a multipotential neural progenitor. These cells appear to be typically restricted by their local brain environment to produce only oligodendrocytes and some astrocytes, in response to local environmental signals whose identities remain to be established. But when removed from the
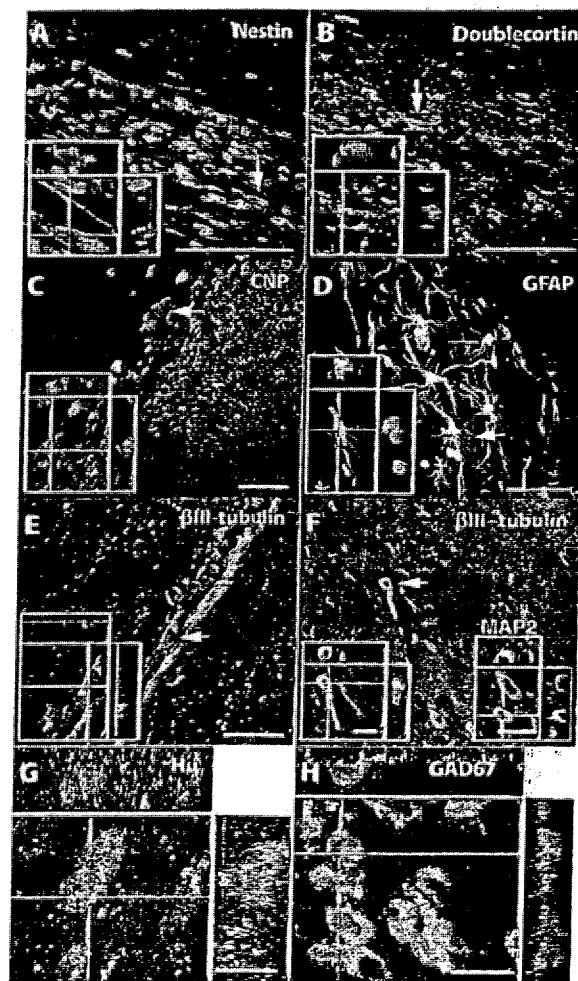

FIGURE 10.4

WMPCs engrafted into fetal rats gave rise to neurons and glia in a site-specific manner. Sections from a rat brain implanted at E17 with A2B5-sorted WMPCs and sacrificed a month after birth. These cells were maintained in culture for 10 days prior to implant. (A–B) Nestin$^+$ (red) progenitors and doublecortin$^+$ (red) migrants, respectively, each co-expressing human nuclear antigen (hNA, green) in the hippocampal alvius. (C) CNP$^+$ oligodendrocytes (red) that were found exclusively in the corpus callosum. (D) A low-power image of GFAP$^+$ (green, stained with anti-human GFAP) astrocytes along the ventricular wall. (E) βIII-tubulin$^+$ (green)/hNA$^+$ (red) neurons migrating in a chain in the hippocampal alvius. (F) βIII-tubulin$^+$ and MAP2$^+$ (inset in part F) neurons in the striatum, adjacent to the RMS (antigens in green; hNA in red; yellow: double-stained human nuclei). (G) An Hu$^+$/hNA$^+$ neuron in the septum. (H) An hNA$^+$ (green)/GAD-67$^+$ (red) striatal neuron. Insets in each figure show orthogonal projections of a high-power confocal image of the identified cell (arrow). From Nunes *et al.* (2003). Scale: A–E, 40 μm; F–H, 20 μm.

environment of the brain and from other brain cells, these cells proceed to make all brain cell types, including neurons and glia, and remain able to do so for long periods of time in culture.

This observation has precedent in lower species. Progenitor cells capable of giving rise to multiple lineages, including oligodendrocytes and neurons, have been consistently derived from the cortical and subcortical parenchyma as well as from the ventricular zone of embryos (Davis and Temple, 1994; Qian et al., 1997; Williams et al., 1991). Similar multipotential progenitors have shown to exist in early postnatal rat cortex (Marmur et al., 1998). A more recent study suggested that postnatal rat optic nerve derived O-2A progenitor cells could be "reprogrammed" to multipotential stem cells capable of generating neurons (Kondo and Raff, 2000). This was achieved by sequential exposure of O-2A progenitors to serum to induce astrocytic differentiation, followed by their expansion in the presence of bFGF in serum-free conditions. Constant mitogenic stimulation of adult rat forebrain parenchymal cells with FGF2 has been shown to result in the generation of neurons as well as astrocytes and oligodendrocytes (Palmer et al., 1995; Richards et al., 1992). Together, these observations of the multilineage potential of CNS glial progenitors suggest that the apparent lineage commitment of progenitors might depend on epigenetic factors. As a result, the nominally glial progenitors of the adult white matter may retain far more lineage plasticity and competence than traditionally appreciated. Adult subcortical P/CNP2:hGFP$^+$ progenitors, though competent to generate multiple cell types, may therefore be restricted to the oligodendrocytic lineage by virtue of the epigenetic bias imparted by their environment before their isolation.

A corollary of the environmental restriction of WMPC phenotype is that other, non-white-matter-derived neural progenitors might similarly restrict to oligodendrocytic lineage when presented to the environment of the adult white matter. Indeed, several groups have reported that EGF-expanded murine neural stem cells differentiate as oligodendrocytes upon xenograft (Mitome et al., 2001); remarkably, in none of these models were substantial numbers of oligodendrocytes generated in vitro. Similarly, v-myc transformed neural stem cells transplanted to perinatal mice can differentiate as oligodendrocytes once recruited to the white matter (Yandava et al., 1999), but not otherwise, and never in vitro.

The Distribution and Heterogeneity of White Matter Progenitor Cells

The persistence and sheer abundance of WMPCs in the adult human brain is striking: Over 3% of the white matter cell population may be sorted on the basis of CNP2:GFP-based FACS, and over half of these cells are mitotically active upon isolation (Roy et al., 1999). That being said, the extent to which this parenchymal progenitor cell population is homogeneous remains unclear; by limiting dilution analysis, only 0.2% of its cells are multipotential (Nunes et al., 2003). Nonetheless, the very existence of multipotential progenitors scattered throughout the white matter parenchyma forces us to reconsider our understanding of both the nature and incidence of neural stem cells in the adult brain and challenges our conception of the supposed rarity of adult neural progenitor and stem cells. In doing so, they point to an abundant and widespread source of cells, which may be used both as a target for pharmacological induction and as a cell type appropriate for therapeutic engraftment to the diseased adult brain.

THERAPEUTIC POTENTIAL OF HUMAN OLIGODENDROCYTE PROGENITOR CELLS

The Natural History of Remyelination in the Adult CNS

The existence of active remyelination in the adult human brain has been mainly derived from observations of MS lesions. However, it has been unclear whether that remyelination has been the result of local expansion of parenchymal OPCs or of the recruitment of distant OPCs to sites of acute demyelination. Moreover, the source and in resting phenotype of the

remyelinating cells has been unclear. To address these questions, Gensert and Goldman (1997) used a combination of retroviral labeling and lysolecithin-induced demyelination to show that normally cycling cells of the adult rodent WM can differentiate as myelinating oligodendrocytes (Gensert and Goldman, 1997). Interestingly, before the endogenous OPCs participated in remyelination, they proliferated locally. Similarly, mice infected with a demyelinating murine hepatitis virus exhibited almost a 14-fold increase in PDGF-αR$^+$ OPCs in the lesion bed (Redwine and Armstrong, 1998). Other studies using rats with EAE or ethidium bromide lesions have shown that after remyelination, OPC numbers were stable (Levine and Reynolds, 1999). This in turn suggested that OPCs can undergo asymmetric division to replicate themselves while generating a differentiating oligodendrocyte.

There appears to be limited survival of OPCs in demyelinated lesions; as a result, most remyelination may be accomplished by unaffected OPCs recruited from the lesion surround. Carroll *et al.* have shown that OPCs in regions adjacent to immunolytic lesions first respond by dividing, followed by their migration into the lesion, and ultimate myelinogenesis (Carroll *et al.*, 1998). Similar observations were made in the demyelinated adult spinal cord, where the population of NG2$^+$ cells expanded significantly in areas adjacent to demyelinating lesions. In this case though, the proliferating pool appeared unable to sustain its self-renewal, as NG2$^+$ cells were depleted following remyelination (Keirstead *et al.*, 1998). Using X-irradiation, Chari and Blakemore (2002) reported that locally recruited NG2$^+$ and PDGF-α R$^+$ OPCs can repopulate depleted areas over distances of approximately 0.5 mm per week in the first month. No secondary progenitor loss was observed in those surround regions from which progenitor cells were recruited, indicating dynamic replacement of the emigrants (Chari and Blakemore, 2002). However, the question of how far the progenitor population can migrate in intact tissue remains debatable, an issue of particular concern for remyelination strategies involving transplantation (Franklin and Blakemore, 1997). Complicating matters further, recent studies have reported an age-related decrease both in recruitment of OPCs and in their subsequent differentiation (Sim *et al.*, 2002).

Candidate Cellular Vectors for Experimental Remyelination via Progenitor Implantation

Progenitor cells capable of local cell genesis therefore persist throughout the subcortical white matter of the adult brain, where they might constitute a potential substrate for cellular replacement and local repair. However, several criteria must be considered when evaluated the transplantation potential of any progenitors. These include the ability of transplanted cells to survive in the host environment, to migrate accurately to the target lesion or tissue type, to generate myelin, to ensheath host axons, and to achieve a degree of myelination capable of functional reconstitution. To assess the myelinogenic potential of transplanted cells, a variety of cell types including multipotential stem cells and OPCs, derived from both animals and humans, have been tested in both developmentally dysmyelinated and experimentally demyelinated models of myelin loss.

Neural Stem Cells and Progenitors from the Fetal Brain

Human fetal brain cells have been found to have robust myelinogenic capacity in the *shiverer* mice (Gansmuller *et al.*, 1986; Gumpel *et al.*, 1987, 1989). Cells isolated from the rodent or human fetal forebrains at various gestational ages, and expanded *in vitro* under a variety of serum-free, factor-supplemented conditions, have been used as sources of transplantable cells (Ader *et al.*, 2001; Brustle *et al.*, 1998; Carpenter *et al.*, 1999; Fricker *et al.*, 1999; Hammang *et al.*, 1997). However, there are potential risks to prolonged *in vitro* expansion, since the cells are not only exposed to exogenous mitogens, but also to autocrine factors in artificially high concentration, and to paracrine agents produced by the neurons and glia present within the initially mixed cultures. As a result, propagated stem or progenitor cells may not retain or reflect the lineage potential or differentiation competence of the native progenitor cells from which they derived. Two recent studies have highlighted the effects of *in vitro* expansion of cells prior to transplant. Buchet *et al.* observed that freshly isolated cells

proliferated longer and gave rise to very extended grafts before differentiating into neurons and glia while cells that were expanded prior to transplant showed poor proliferation and quick differentiation capacity (Buchet *et al.*, 2002). In contrast, Englund *et al.* found that after 9 weeks of expansion, human fetal brain cells lost the capacity to differentiate and remained as undifferentiated progenitors when transplanted into adult recipients (Englund *et al.*, 2002). To circumvent the issue of paracrine effects on defined stem cells in mixed culture, several groups have developed methods of directly isolating neural stem cells from tissue, thereby preventing their *in vitro* exposure to differentiated cell products during either isolation or expansion (Keyoung *et al.*, 2001; Uchida *et al.*, 2000).

Neural Stem Cells and Progenitors from Adult Brain

Several studies describe the use of neural stem cells derived primarily from the adult rat and human VZ, and then propagated as neurospheres, as a potential source of myelinogenic cells (Akiyama *et al.*, 2001; Kukekov *et al.*, 1999; Zhang *et al.*, 1999). As described earlier, the adult human white matter harbors an abundance of oligodendrocyte progenitors. By virtue of their abundance, these progenitors represent a potential cellular substrate for therapeutic transplantation. Nonetheless, only a few studies, constrained by the lack of any reliable method to isolate these cells, have attempted to assess the myelinogenic capacity of OPCs derived from the adult human white matter. In one such study (Targett *et al.*, 1996), a crude cell preparation derived from adult human white matter was transplanted into the ethidium bromide-lesioned and radiosensitized, X-irradiated adult rat spinal cord. The transplanted oligodendrocytes survived in the demyelinated zone, associated with denuded host axons, and expressed myelin proteins. But the transplanted cells did not migrate or divide, nor was any myelination noted. The failure of these implanted oligodendrocytes to myelinate was attributed to the diminished regenerative potential of post-mitotic oligodendrocytes, and the lack of a permissive environment for remyelination within the rat lesion bed (Targett *et al.*, 1996).

Propagated Oligospheres

Though neural stem cells have myelinogenic capacity, they also have the inherent capacity to generate neurons and astrocytes. The co-generation of astrocytes may not necessarily be deleterious, given their roles in both OPC proliferation and myelination (Blakemore, 1992; Franklin *et al.*, 1991). However, the co-generation of neurons may be undesirable, given the potential generation of ectopic neuronal foci, which might conceivably act as epileptogenic foci. Thus, priming neural stem cells or OPCs toward oligodendrocytic differentiation prior to implant might be necessary to ensure the quantities and phenotypic homogeneity of oligodendrocyte progenitor cells that will be needed for clinical implantation. One approach to this goal has been the expansion of neural stem cells as neurospheres in the presence of oligodendrocyte-inducing agents. For instance, when rat cerebellum-derived neurospheres were propagated in the presence of conditioned medium from the neuroblastoma B104 line (B104/CM), oligodendrocytes were preferentially generated. The resultant "oligospheres" were capable of being exponentially expanded through several passages without phenotypic degradation and exhibited robust myelination on transplantation into the *shiverer* mice brain (Avellana-Adalid *et al.*, 1996). Since then, several groups have used a similar strategy to generate oligospheres from neural precursor cells of the mouse, rat, and canine forebrains (Vitry *et al.*, 1999; Zhang *et al.*, 1998). Smith and Blakemore the compared the remyelinating capacity of cells isolated from porcine SVZ within hours after dissociation, to that exhibited by matched cells after growth in B104/CM as oligospheres. Whereas the freshly isolated SVZ cells remained undifferentiated after xenograft, those expanded in B104/CM effected significant remyelination of demyelinated axons *in vivo* (Smith and Blakemore, 2000).

Human OPCs Integrate When Grafted to Demyelinated Foci of the Adult Rat Brain

The remyelinating potential of adult human white matter-derived progenitors has been recently shown in lysolecithin-induced demyelinating lesions of adult rat corpus callosum

(Windrem *et al.*, 2002). In this study, A2B5 expression by P/CNP2:hGP-defined OPCs (Roy *et al.*, 1999) provided the rationale for immunomagnetically selecting OPCs on the basis of A2B5 expression. Like P/CNP2:hGFP⁺ cells, A2B5-sorted cells generated largely oligodendrocytes when raised at high density in the presence of serum. In addition, immunomagnetic selection allowed their higher-yield acquisition, without the losses in viability and number associated with FACS separation. As a result, A2B5-antibody based immunomagnetic sorting increased the yield of extractable OPCs by over 5-fold. These A2B5-sorted white matter progenitors were transplanted into cyclosporine-immuno-suppressed adult rats, 3 days after lysolecithin lesions. As previously described (Gensert and Goldman, 1997), these lesions yielded a discrete region of transcallosal demyelination, with mild local reactive astrocytosis within the demyelinated focus, and intact vasculature. When A2B5-sorted human OPCs were injected into these lesions, they migrated widely and rapidly; within 7 days of implantation, the cells had readily traversed the midline to infiltrate the furthest reaches of the demyelinated lesion beds, which often extended over 8 mm in breadth. The migration rate of the cells was hence at least 1 mm/day, or 50 µm per hour, within the lesion borders (Fig. 10.5). The engrafted adult A2B5-sorted progenitors differentiated rapidly, expressing CNP within 2 weeks and MBP within 3 weeks of implant-ation. These OPC-derived oligodendrocytes projected MBP⁺ lamellopodia and were asso-ciated with a branched array of myelinating fibers, indicating the initiation of progenitor-associated myelinogenesis. Of note, many transplanted progenitor derived astrocytes were also observed in the lesions. With cyclosporine immunosuppression, the cells could survive at least 2 months in lysolecithin-demyelinated recipients. These findings suggested that the introduction of highly enriched preparations of progenitor cells derived from the adult human white matter might permit local remyelination.

Migratory Characteristics of human OPCs

Adult human-derived OPCs engrafted into demyelinated brain remained restricted to regions of demyelination; they rarely extended into normal surrounding myelin (Fig. 10.5). Even the few cells that were typically noted to have infiltrated normal myelin appeared to have migrated along the extraluminal surfaces of penetrating blood vessels. Yet when lentiviral-GFP tagged A2B5-sorted progenitor cell pools from adult human white matter were implanted into intact subcortex of adult rats, the transplanted cells remained localized to the implant site and continued to be so even after 3 months (Windrem *et al.*, 2002). These observations suggest strongly that normal adult white matter is non-permissive for the migration of adult-derived WMPCs, as has been observed in other studies (Iwashita *et al.*, 2000). This restriction on migration may be similar at the molecular level to that observed toward axons, whose extension through normal white matter is suppressed by their expression of Nogo receptor, by which they respond to myelin-associated Nogo and MAG (myelin-associated glycopro-tein) with repulsion and/or cessation of further advance (Grandpre and Strittmatter, 2001). That being said, the operative white matter signals that restrict progenitor cell migration have yet to be identified. Whatever its mechanism, normal myelin clearly retains cues sufficient to tonically impede WMPC infiltration; accordingly, demyelination appears to remove those cues, allowing the active invasion and dispersion of OPCs throughout regions of acute myelin loss. The characterization of the ligands providing these repulsive cues, and of their antici-pated progenitor cell receptors, will likely constitute an important avenue of future study.

Myelin Construction by Perinatal Transplant-Based Therapy

Several models of congenital dysmyelination have been used to assess the myelinogenic potential of animal and human-derived progenitors. The myelinogenic potential of im-planted fetal human brain cells was first noted in the shiverer mouse (Gumpel *et al.*, 1987; Lachapelle *et al.*, 1983). The myelinogenic potential of different, stage-defined phenotypes of oligodendrocyte progenitors, extracted so as to sample the engraftment efficacy of different stages of progenitor progression, have also been compared in shiverer mice. Using rat donor tissue, Warrington and Pfeiffer established that the A2B5-defined oligo-
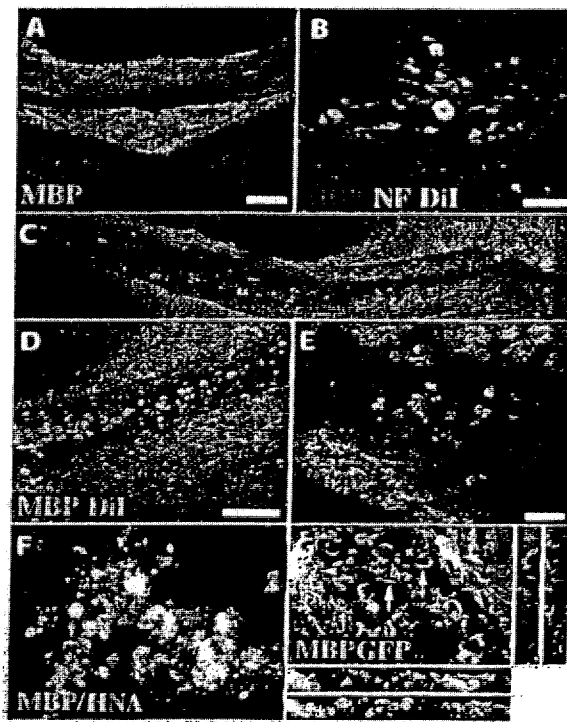
FIGURE 10.5

Implanted white matter progenitors migrated widely throughout the demyelinated callosum. Sorted adult human white matter progenitors were transplanted into lysolecithin-induced demyelinated lesions in the corpus callosa of adult rats. (A) Lysolecithin infusion (1 μl of 2% lysolecithin-V, delivered into the corpus callosum) yielded demyelinated plaques in the target white matter. In part A, the lesion is visible as a discoid region of myelin basic protein (MBP)-immunonegativity, surrounded by the otherwise MBP[+] callosum (green). (B) Though denuded of myelin (MBP, blue), neurofilament[+] axons (green) initially survived lysolecithin demyelination, 1 week after callosal lesion. The implanted progenitors (orange) have just immigrated to the lesion bed. Axonal spheroids were frequent within the lysolecithin-lesion bed, indicating some degree of early injury and transection. The ability of implanted progenitors to effect repair is limited by the viability and integrity of the axonal cohort that one wishes to myelinate. (C) This low-power montage demonstrates the rapidity of long-distance migration by xenografted adult human white matter progenitors. These DiI-labeled human progenitor cells (red) were visualized 1 week after their implantation, by which point the cells extend throughout the demyelinated lesion, defined by its loss of myelin basic protein (MBP)-immunoreactivity (green). The lesion was induced 3 days before 10[5] sorted, DiI-tagged (red) human progenitors were delivered in 2 μl. Within a week of implantation into this demyelinated callosum, the cells had traversed the midline. (D) A higher magnification image showing that the transplanted cells migrated throughout the demyelinated plaque, but not beyond its borders, except for occasional migrants that followed the parenchymal surfaces of blood vessels (arrow). The restriction of migration to demyelinated regions suggests that normal myelin impedes the migration of these cells. (E) Human white matter progenitor cells, identified as human nuclear antigen[+] (HNA; green), occupied the MBP (green)-deficient lysolecithin lesion, and expressed oligodendrocytic CNP (red) by 15 days after implantation. (F) A cluster of HNA^{+} human cells (green) associated with a plethora of donor-derived MBP[+], myelinating oligodendrocytic lamellopodia (red). (G) Lentiviral GFP-tagged human (green) MBP[+] (red) oligodendrocytes in the lesion bed of a lysolecithin-injected rat callosum, 8 weeks after cell implantation. Besides the MBP^{+} cells (arrows), other human progenitor-derived cells were also present, which did not express MBP and which instead manifested astrocytic morphologies (arrowheads). Immunolabeling adjacent sections for human GFAP (red) revealed that many of GFP-tagged human progenitors had also given rise to astrocytes. From Windrem et al. (2002). Scale bars: A, 200 μm; B, 20 μm; D, 100 μm; E, 30 μm.

dendrocyte progenitor phenotype was more efficient at migration and myelinogenesis in neonatal shiverers than the more mature O4-defined oligodendrocyte (Warrington *et al.*, 1993). Yandava *et al.* similarly achieved myelination within the shiverer brain, using the C17.2 line of transformed murine cerebellar progenitor cells, which act as neural stem cells after v-myc immortalization (Yandava *et al.*, 1999).

Similarly, fetal oligodendrocytes transplanted to the *md* rat remyelinated significant portions of the postnatal spinal cord (Archer *et al.*, 1994). Moreover, analogous studies in the *shaking* pup showed that fetal oligodendrocytes were able to engraft widespread regions of the *shaking* CNS, with graft survival of over 6 months. Although neonatal recipients fared best, adult recipients also exhibited graft oligodendrocyte survival and stable myelination (Archer *et al.*, 1997). Duncan and colleagues then demonstrated that oligosphere-derived cells raised from the neonatal rodent subventricular zone could engraft another dysmyelinated mutant, the myelin-deficient rat, upon perinatal intraventricular administration (Learish *et al.*, 1999). The success of these approaches led then to the seminal work of Mitome and colleagues, who used EGF responsive primary neural progenitor cells, in tandem with a combination of ventricular and cisternal transplant, to achieve the widespread myelination of the shiverer brain (Mitome *et al.*, 2001).

Human OPCs Can Myelinate Congenitally Dysmyelinated Brain

On the basis of these studies, Windrem *et al.* investigated whether highly enriched populations of human progenitor cells, directly isolated from the brain, might be used for cell-based therapy of congenital dysmyelination. Specifically, this study postulated that the efficiency of myelination might be improved by using purified OPCs, derived via selection so as to exclude astrocytes, microglia, and vascular derivatives from the implanted pool. It further postulated that such purified human OPCs, both adult-derived and taken from the fetal brain during its period of maximal oligoneogenesis, would be sufficiently migratory and myelinogenic to mediate the widespread myelination of a perinatal host. To this end, A2B5-based FACS was used in conjunction with PSA-NCAM-dependent immuno-depletion of neuronal derivatives, to prepare highly enriched dissociates of human OPCs, of both fetal and adult derivation. Both classes of human oligodendrocyte progenitor cells proved capable of widespread and high-efficiency myelination of the shiverer brain after perinatal xenograft. Indeed, the cells migrated so widely as to effect myelination through-out the recipient brains (Fig. 10.6, unpublished data). The cells infiltrated widely throughout the presumptive white matter, ensheathed resident murine axons, and formed antigenically and ultrastructurally compact myelin. After implantation, the cells slowed their mitotic expansion with time and generated neither undesired phenotypes nor paren-chymal aggregates. In this initial study, despite histologically extensive myelination in these animals, no change in the behavioral phenotype of the shi/shi recipients or any improve-ment in their neurological phenotype was evident. Nonetheless, the geographic extent of forebrain and diencephalic MBP expression evidenced by these animals, who received but a single perinatal intraventricular cell injection, suggested that combined cisternal and intraventricular delivery of donor progenitors might achieve remyelination throughout the rostral neuraxis, potentially spanning the entire brain.

Besides demonstrating the myelinogenic capacity of the transplanted cells, studies in the dysmyelinated animal models indirectly indicate that congenital dysmyelination, even more so than adult demyelination, may be an appropriate target for CNS progenitor cell-based therapy. In particular, these studies affirmed that the neonatal brain environ-ment may be especially amenable to therapeutic remyelination. It is conducive to wide-spread migration and may continue to provide the instructive developmental cues necessary for region-specific differentiation.

Fetal and Adult OPCs Differ

Despite the use of both fetal and adult-derived OPCs in experimental therapeutic models, no head-to-head comparison of the two phenotypes had ever been performed from
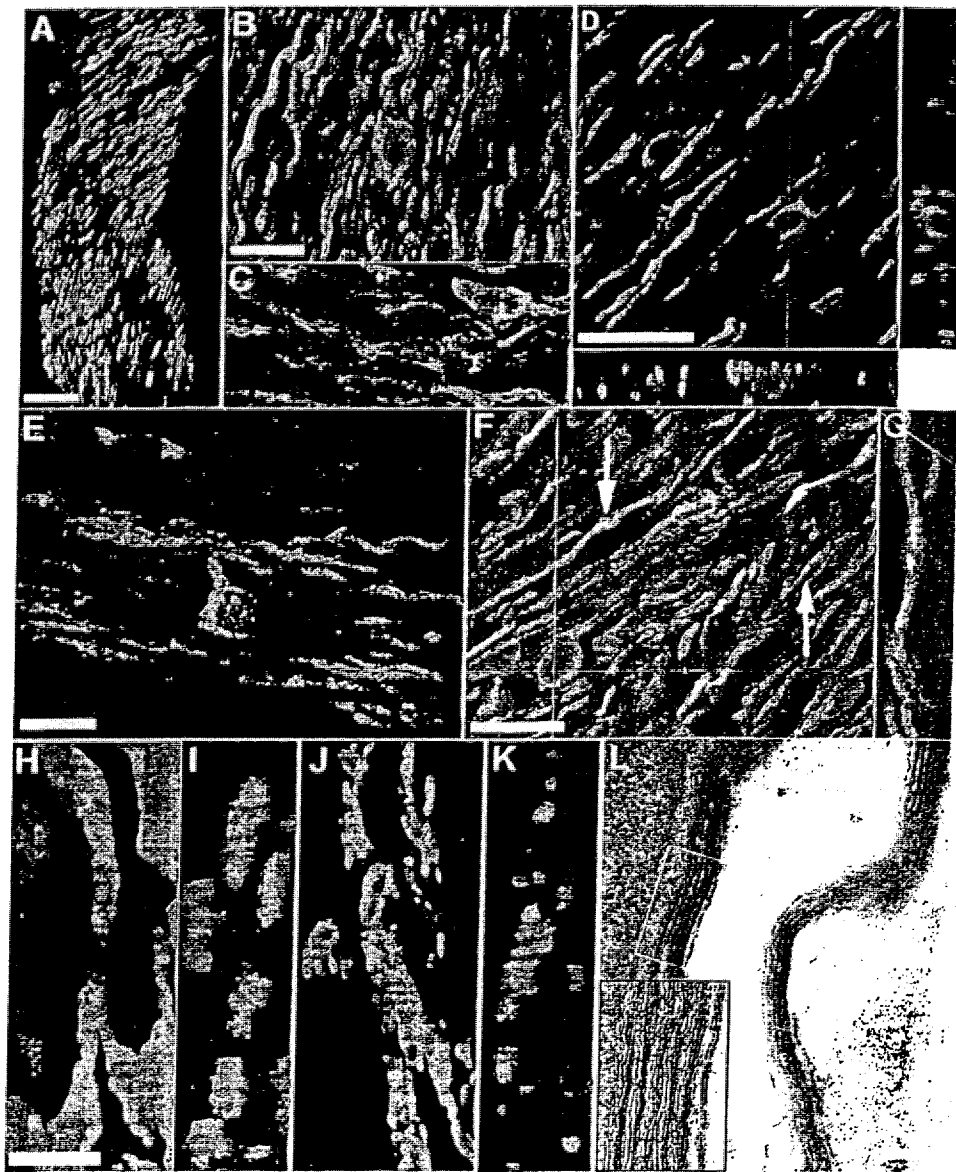
FIGURE 10.6

Myelin basic protein (MBP) was widely expressed by human fetal OPCs implanted into neonatal homozygote shiverer mice. (A) This low-power view of the recipient fimbria shows abundant fiber-associated MBP expression (green), 3 months after perinatal engraftment (MBP, green) Since shiverer homozygotes do not express immunoreactive MBP, all such signal must derive from donor progenitor cells. (B-D) Donor fetal OPCs, additionally validated as such by human nuclear antigen (hNA, red), differentiated to express CNP protein (green in B) and MBP (green in C-D). In the 0.5 µm confocal optical section of D, MBP (green) is noted to surround the donor human nucleus (red), as viewed in orthogonal planes. (E). A single donor-derived MBP⁺ oligodendrocyte that has matured, 3 months after engraftment, to associate with multiple recipient axons. (F) An 0.2 µm optical section through a recipient corpus callosum shows engrafted human OPCs (hNA, blue) expressing MBP (red), and surrounding native axons (neurofilament, green). Arrows indicate examples of ensheathed axons, a higher magnification of which is shown in (G). Human OPCs enwrap native axons and reorganize the paranodal region to permit nodal formation. Caspr protein, an axonal paranodal marker, is expressed on unmyelinated axons between myelinated segments of axon, without invading the nodal region. (H-K) Optical sections through engrafted shiverer corpus callosum, showing donor-derived MBP (green) and native axonal Caspr protein in red, indicating that donor OPCs develop not only myelin production and architecture, but permit nodes of Ranvier to form (anti-Caspr antibodies generously provided by Dr. M. Rasband). (L) Electron microscopy confirmed that donor-derived oligodendrocytes developed compact myelin, I that myelin produced by engrafted fetal human OPCs wrapped native axons to form compact sheathes with major dense lines (inset). Scale bars: A, 200µm; B-F, 20µm; H-K, 5µm; L, 1 µm.

analogously acquired and maintained cells implanted into the same models at the same time. As a result, it was unclear if fetal-derived OPCs differed from their counterparts derived from the adult human brain, with respect to either their migration competence, myelinogenic capacity, or the time courses thereof. To assess the relative advantages and disadvantages as therapeutic vectors of these two stage-defined OPC phenotypes, newborn shiverer mice were implanted with either fetal or adult-derived OPCs, each isolated via A2B5-directed immunomagnetic sorting (IMS). The implanted neonatal mice were allowed to survive for 1 to 3 months, and their brains then sectioned and stained for MBP, GFAP, and anti-human nuclear antigen. By this means, it was determined that fetal and adult-derived human OPCs differed substantially in their respective time courses and efficacy of myelinogenesis upon xenograft. Adult OPCs myelinated shiverer brain more rapidly than their fetal counterparts, achieving widespread and dense MBP expression by 4 weeks after xenograft. In contrast, substantial MBP expression by fetal OPCs was generally not observed until 12 weeks post-implant (Windrem *et al.*, unpublished data).

Besides myelinating more quickly than fetal OPCs, adult OPCs were found to give rise to myelinogenic oligodendrocytes in much higher relative proportions, and with much less astrocytic co-generation, than did fetal-derived OPCs. When assessed at the midline of the recipient corpus callosum, just over 10% of fetal hNA-defined OPCs expressed MBP at 12 weeks, while virtually none had done so at 4 weeks. In contrast, almost 40% of adult OPCs expressed MBP by 4 weeks after xenograft into matched recipients. Thus, engrafted adult OPCs were at least four times more likely to mature as oligodendrocytes and develop myelin than their fetal counterparts. As another cardinal difference between fetal and adult OPCs, adult OPCs largely remained restricted to the host white matter, within which they generated almost entirely MBP$^+$ oligodendrocytes. In contrast, fetal OPCs migrated into both gray and white matter, generating both astrocytes and oligodendrocytes in a context-dependent manner.

Thus, both fetal and adult-derived OPCs were competent to remyelinate murine axons, but each had distinct advantages and disadvantages as potential vectors for cell therapy: Whereas fetal OPCs were highly migratory, they myelinated slowly and inefficiently. In contrast, adult-derived OPCs migrated over lesser distances, but they myelinated more rapidly and in higher proportions than their fetal counterparts. Together, these studies argued that while both fetal and adult human OPCs might provide effective cellular substrates for remyelination, the choice of cellular source must be dictated not only by the availability of donor material, but also by the specific biology of the disease target.

A Caveat: Some Implanted Progenitors May Remain Undifferentiated

A corollary of the multipotential nature of white matter progenitor cells is that when transplanted as nominally oligodendrocytic precursors, these cells might encounter local signals that instruct their maturation along alternative lineages. As a result, we need to be concerned about the possibility of their differentiation into undesired or functionally heterotopic phenotypes. This possibility is of further concern given the persistence of many implanted progenitors as undifferentiated cells; these may remain able to respond to signals in the host tissue environment, not only at the time of implantation, but also long thereafter. As such, these cells might comprise a reservoir of implanted precursors, from which desired phenotypes might be later recruited upon injury or insult. On the other hand, they might just as well constitute potential sources of undesired cell types that might be ectopically generated and recruited in the tissue environment of an acutely injured focus. Such local production of undesired phenotypes might introduce not only inefficiency to transplant-based treatment strategies, but also frank danger. For instance, the production of neurons in a white matter lesion could generate an epileptogenic focus, just as the production of astrocytes in a more typically oligodendrocytic region might disrupt local ionic gradients and hence axonal transmission. These and many other untoward processes of heterotopic phenotypic maturation could more than offset whatever benefits might be gleaned from a therapeutic cell implant. As a result, it may prove advisable to initiate the phenotypic differentiation of these cells *in vitro*, prior to implantation, so as to limit

the range of phenotypic choices available to the isolated progenitors to those appropriate for the intended region and disease target. Time will tell whether the possibility of heterotopic misdifferentiation will mandate such *in vitro* priming steps.

EXPERIMENTAL IMPLANTATION OF NON-CNS PROGENITOR CELL TYPES

A wide range of other potentially myelinogenic cell types have also been implanted into experimental models of de- and dysmyelination, with varying degrees of success.

Schwann Cells

Schwann cells, the myelinating cells of the peripheral nervous system, have been considered as an attractive alternative to oligodendrocyte precursors for experimental transplantation. Schwann cells from several sources, including humans (Kohama *et al.*, 2001), have been implanted in dysmyelinated *shiverer* mice (Baron-Van Evercooren *et al.*, 1992), MD rats, and *shaking* pups (Duncan and Hoffman, 1997). They have also been transplanted into lysolecithin (Baron-Van Evercooren *et al.*, 1993; Duncan *et al.*, 1981) and EB-X (Blakemore and Crang, 1985) demyelinated lesions in the brain and spinal cord. In all these systems, they have demonstrated varying degrees of myelination (Franklin and Barnett, 1997) with the myelin produced by these cells being of the PNS-variety as specified by the expression of P0. In some cases, functional reconstitution of saltatory conduction has also been shown (Felts and Smith, 1992; Honmou *et al.*, 1996; Kohama *et al.*, 2001). In addition, Schwann cells have been reported to improve axonal regeneration, which might be of importance in MS where axonal loss is a major part of the lesion pathology. Considering the relative ease of expanding human Schwann cells in culture (Rutkowski *et al.*, 1995), it has been suggested that they might be appropriate cellular vectors for autologous transplants. Indeed, they have the added advantage of producing non-CNS myelin, which may be refractory to the immunological destruction in diseases like MS. However, like central oligodendrocyte progenitors, the migratory capacity of these cells is unclear. Some studies indicate that Schwann cells migrate satisfactorily over large distances to specific target sites (Franklin and Barnett, 1997), while others indicate that they are unable to migrate through normal white matter (Iwashita *et al.*, 2000). In addition, Schwann cells seem to have a complex relationship with central astrocytes. After transplantation, Schwann cells are mainly found in areas devoid of astrocytes (Baron-van Evercooren *et al.*, 1992; Blakemore and Patterson, 1975), and, moreover, they are excluded as astrocyte numbers increase with time (Shields *et al.*, 2000).

Olfactory Ensheathing Cells (OEC)

In nature, OEC's ensheath small diameter axons of the peripheral olfactory epithelium neurons that project through the olfactory nerve into the olfactory bulb of the CNS. Unlike Schwann cells, these cells do not normally produce myelin. However, OECs from both animal (Franklin *et al.*, 1996; Imaizumi *et al.*, 1998) and human sources (Barnett *et al.*, 2000; Kato *et al.*, 2000) show remyelination with a peripheral pattern of myelin expression upon transplantation to demyelinated spinal cords. In some studies, a functional restoration of conduction has also been demonstrated (Imaizumi *et al.*, 2000). OECs may have an advantage over Schwann cells, as they co-exist naturally with astrocytes in the olfactory bulb (Lakatos *et al.*, 2000). Perhaps as a result, their interaction with astrocytes in not restrictive (Franklin and Barnett, 2000) in fact, they have been reported to support axonal regeneration through the astrocytic environment of a transected spinal cord (Ramon-Cueto *et al.*, 1998). Nonetheless, their restoration of central axonal conduction remains inconclusive, as is the long-term fate of their remyelinated units. Whether these cells are capable of the contact-dependent and humoral support of neuronal function

normally exercised by central oligodendrocytes, or conversely, whether they are in turn supported by the axons with which they interact (Fernandez et al., 2000; Vartanian et al., 1997), similarly remain unknown.

Embryonic Stem Cells

Myelination by *in vitro* conditioned mouse embryonic stem cells has been reported in both hypomyelinated MD rat E-17 fetuses and *shiverer* newborns, as well as in adult lysolecithin demyelinated lesions in adult rats (Brustle et al., 1999; Liu et al., 2000). More recent reports describe transplanted human ES cells sequentially cultured to induce neural stem cells capable of generating oligodendrocytes in a region-specific manner (Reubinoff et al., 2001). Though ES cells might represent a readily cultivable source of OPCs, the use of these cells is still limited by our inability to fully instruct all cells in the undifferentiated population to the desired phenotype. Of greater concern is the persistent uncommitted progenitors within the implanted population, which may retain the latent capacity for undifferentiated expansion and possibly tumorigenicity.

Mesenchymal and Marrow-Derived Stem Cells

In addition to ES cells, mesenchymal and marrow-derived stem cells have been in focus as a source of neurally specified cells. Some controversial studies indicate that these cells may be capable of trans- or ectopic differentiation to neuroectodermal lineage (Mezey et al., 2000; Sanchez-Ramos et al., 2000). Of particular concern has been the lack of clear clonal evidence of neural specification as well as recent reports indicting that cell fusion may explain some of observations of trans-differentiation (Terada et al., 2002; Ying et al., 2002). Nonetheless, a recent study, in which mouse bone marrow stromal cells were grafted into EB-X demyelinated spinal cord lesions, reported not only donor-cell derived histological remyelination, but also an improvement in conduction velocity (Akiyama et al., 2002). This work remains to be replicated by other groups. Should this study prove verifiable, its approach may open new avenues of stromal cell-based remyelination therapy.

DISEASE TARGETS FOR PROGENITOR-BASED THERAPEUTIC MYELINATION

Congenital Dysmyelination

Congenital diseases of myelination, such as periventricular leukomalacia (PVL), which may serve as an anatomic form fruste for the later development of cerebral palsy (Grow and Barks, 2002; Rezaie and Dean, 2002; Volpe, 2001) and the hereditary leukodystrophies and storage diseases, such as Krabbe's and Tay Sachs disease, are leading causes of infant morbidity and mortality (reviewed by Schiffmann and Boespflug-Tanguy, 2001; Berger et al., 2001). As such, these may constitute feasible and attractive targets for therapeutic remyelination (Tate et al., 2001).

Periventricular leukomalacia PVL describes a lesion of the periventricular white matter, associated with a failure in early myelination of the cerebral hemispheres. PVL appears to be a pathological concomitant to perinatal hypoxic-ischemic insult and may result from germinal matrix hemorrhage, sustained hypoxia, and excitotoxic injury, and most likely from combinations of these insults. PVL predicts the development of cerebral palsy in most cases (Volpe, 2001). Experimental models of hypoxic-ischemia in neonatal rats (Back et al., 2002; Levison et al., 2001) as well as studies of pediatric autopsies (Back et al., 2001) have suggested that the late oligodendrocyte progenitors of the forebrain SVZ comprise the predominant cell population lost in perinatal ischemic injury. This is in accord with our understanding of the natural history of oligodendrogliogenesis in humans (Grever et al., 1999; Rakic and Zecevic, 2003; Zhang et al., 2000). the developmental window for which corresponds to the period of ischemic vulnerability of the periventricular white matter.

Congenital leukodystrophies include an ever-expanding group of inherited diseases of myelin synthesis and metabolism. Although a diverse group, these may roughly be divided into lysosomal storage diseases, such as Krabbe's globoid cell leukodystrophy (Wenger *et al.*, 2000) and Tay Sachs diseases (Gravel *et al.*, 1991); disorders of myelin synthesis, such as Pelizaeus-Merbacher disease (PMD) (Koeppen and Robitaille, 2002); and metabolic deficiencies leading to toxic demyelination, such as Canavan's disease (Matalon and Michals-Matalon, 2000). Each of these disease categories is attended by extensive white matter involvement and clinical leukoencephalopathy, typically leading to severe neurological disability and death. As a group, the clinical leukodystrophies represent especially attractive targets for progenitor cell-based therapy, since the restoration of healthy oligodendrocytes in early perinatal development may be sufficient to permit myelination and hence to slow or prevent the development of the disease phenotype. In addition, effective murine models of these diseases are available (Werner *et al.*, 1998). Inherited diseases of the PLP and MBP genes are modeled by twitcher (Mikoshiba *et al.*, 1985; Yoshimura *et al.*, 1989) and shiverer mice (Roach *et al.*, 1985), respectively. In addition, mutations of hexoseaminidase-B, modeling Sandhoff's and Tay-Sachs diseases (Kolter and Sandhoff, 1998), and aspartoacylase, mimicking Canavan's disease (Matalon *et al.*, 2000), have been similarly employed. The availability of such genetically precise models of the childhood leukodystrophies is already greatly accelerating the process of developing experimental treatment strategies for these disorders.

Acquired Demyelination

In adults, the diseases of acquired demyelination include later-onset leukodystrophies, such as metachromatic leukodystrophy and adrenoleukodystrophy, as well as vascular, inflammatory, and nutritional demyelinating syndromes (Baumann and Turpin, 2000; Berger *et al.*, 2001; Desmond, 2002; Dichgans, 2002). The vascular demyelinations include hypertensive and diabetic leukoencephalopathies, which may both be due to chronic oligodendrocytic ischemic hypoxia (Dewar *et al.*, 1999; Leys *et al.*, 1999). Subcortical strokes, particularly those within the distributions of the forebrain lenticulostriate and thalamogeniculate arterial territories, are also prominent causes of vascular demyelination (Dichgans, 2002). The inflammatory demyelinations include multiple sclerosis, transverse myelitis (Kerr and Ayetey, 2002), optic neuritis (Cree *et al.*, 2002; Eggenberger, 2001), and less commonly Schilder's leukoencephalitis (Kotil *et al.*, 2002), as well as postvaccinial (An *et al.*, 2002; Konstantinou *et al.*, 2001) and postinfectious leukoencephalitis (Kleinschmidt-DeMasters and Gilden, 2001; Rust, 2000). All of these syndromes of acquired demyelination are potential targets of therapeutic remyelination. Yet most attempts at cell-based remyelination in experimental animals have been made using acute chemical demyelinating insults, such as lysolecithin. In contrast to the availability of effective animal models of congenital dysmyelination, the study of acquired demyelination has suffered from its lack of biologically appropriate, clinically reflective animal models. As a result, few adequate studies of cell-based remyelination of acquired, adult demyelinating lesions have been reported using any cellular vector. Those studies that have reported oligodendrocytic maturation and myelination by implanted oligodendrocyte progenitors have typically failed to demonstrate substantial axonal ensheathment, though this has likely reflected the loss of competent axons in these models, rather than any insufficiency on the part of the implanted progenitor cells. Indeed, the etiological complexity and manifold sequelae of demyelination in the adult brain argues against easy therapeutic intervention. As such, until improved models of acquired demyelinating disease are available, progress in cell-based therapy of adult demyelinating diseases will be necessarily slow. In contrast, the arguably simpler etiologies of congenital dysmyelination, their frequent lack of association with underlying systemic disease, and the persistent structural plasticity of the perinatal brain, together with the many effective animal models for congenital dysmyelination, collaborate to make these diseases attractive targets for near-term intervention, both experimentally and clinically. Indeed, we may reasonably except the congenital leukodystrophies to be especially promising targets for cell-based therapeutic remyelination.

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EVIDENCE APPENDIX
EXHIBIT 18

January 7, 2000, Research/Clinic Update for the National Multiple Sclerosis Society

NATIONAL
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RESEARCH/CLINICAL UPDATE

January 7, 2000

RESEARCHERS FIND KEY CELLS IN ADULT BRAIN THAT MAY SOMEDAY REPAIR MYELIN IN MS

Summary:
- Researchers at Cornell University Medical College, supported by the National MS Society, have for the first time isolated cells in the adult human brain that can divide and grow into myelin-making cells and that may ultimately be capable of replacing those damaged in multiple sclerosis.
- Although very basic in nature, this research may eventually lead to therapies for MS either through implantation of such cells, or through development of ways of stimulating progenitor cells resident in a person's brain to produce new oligodendrocytes that can repair myelin damaged by MS and possibly restore nerve function.

Details: National MS Society-supported investigators led by Steven A. Goldman, MD, PhD, of Cornell University Medical College, have reported the discovery and isolation of a population of immature ("progenitor") myelin-making cells (oligodendrocytes) in the brains of adult humans. These cells have the potential to repair myelin that has been destroyed by MS, and possibly to aid in the recovery of function.

Reporting in the November 15 issue of *The Journal of Neuroscience*, the investigators describe having found that the oligodendrocyte progenitor cells are surprisingly abundant in adult brain matter, and are capable of dividing to produce new oligodendrocytes. Most adult human brain cells do not divide. This is the first demonstration that such cells can be stimulated to divide and give rise to new oligodendrocytes.

Background
Throughout the 1990s, researchers had been searching for oligodendrocyte progenitor cells in the human brain. Oligodendrocyte progenitor cells had been found in the rat brain in the 1970s and 1980s. Immature oligodendrocytes had been found in human

brain tissue, but none of these had been capable of dividing. Researchers had begun to conclude that oligodendrocyte progenitor cells capable of dividing did not exist in the adult human brain. But by using surgically removed samples of adult human brain tissue, combined with newly developed techniques of molecular cell identification and separation, Goldman and colleagues were able to refute this notion and for the first time, segregate a population of dividing oligodendrocyte progenitors in adult brain.

The study
In this study, reported in the November 15 issue of *The Journal of Neuroscience*, adult human brain cells were obtained from brain matter that was removed from eight patients ranging in age from 24 to 65 years old, who underwent surgery for a variety of disorders. The investigators used a technique they had developed and tested in animal brain cells to separate living progenitor cells from the larger brain cell population.

The investigators identified a discrete population of oligodendrocyte progenitor cells, which they estimated to represent about four percent of the population of cells in the in the white matter of the brain. They then segregated the progenitor cells, and demonstrated that they were capable of dividing, "more or less on demand," says Goldman.

What the Study Means
This study shows that oligodendrocyte progenitor cells exist within the adult human brain and are capable of dividing. Furthermore, this study describes a method for the isolation and actual purification of these cells, potentially in large numbers. This raises the possibility that patients with MS might someday be treated either by transplanting oligodendrocyte progenitor cells, or by stimulating the patients' own oligodendrocyte progenitor cells to divide and produce new cells. Treatments might also be devised that combine elements of both approaches.

Dr. Goldman's team is currently conducting studies to determine whether transplanted oligodendrocyte progenitor cells will be able to produce replacement myelin on nerve fibers whose myelin has been destroyed. Studies will also be needed to determine whether stimulating the growth of new oligodendrocytes from progenitor cells that exist within a patient's brain will remyelinate damaged neurons.

The National MS Society is actively funding these and other efforts to find ways to repair myelin and nerve cells that have been destroyed by multiple sclerosis, with the hope of restoring nerve function.

From: Research Programs Department

EVIDENCE APPENDIX
EXHIBIT 19

Summary of MS Research Progress – 1999, National MS Society, December 10, 1999

SUMMARY OF MS RESEARCH PROGRESS - 1999

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December 10, 1999

This has been another exciting year for MS research. Thanks to funds provided by its chapters and private donors, the National Multiple Sclerosis Society was able to spend a record $22.5 million to support research programs in 1999. Since its founding 53 years ago, the Society has invested more than $260 million to find the cause, treatments and cure for MS.

During the year, our volunteer scientific advisors reviewed 300 MS research proposals and approved 129 as being of high scientific merit and relevance and thus warranting the Society's support. The Society now has over $40 million in current and future commitments to over 300 MS research projects, for which money must be raised.

Significant advances have been made in both laboratory and clinical studies in MS. As the world's largest private supporter of MS research, the Society has been at the core of many of these advances during 1999. Key highlights include:

- In separate Society-supported studies, investigators reported evidence for the possibility that human herpes virus-6 (University of Wisconsin) and the bacterium Chlamydia pneumoniae (Vanderbilt University) may be linked to MS. Further studies are ongoing to determine whether either these or other infectious agents are causal factors in MS, and whether drugs to fight these agents can help MS.

- The first large-scale clinical trial of Copaxone in primary-progressive MS was begun. It will eventually enroll 900 people at 54 centers across the U.S. and Canada.

- Society-supported investigators at Cornell University Medical College reported, for the first time, being able to isolate immature ("progenitor") myelin-making cells in the adult human brain, remove them surgically and transform them, in laboratory dishes, into mature cells capable of making new myelin. This important step may provide a basis for new strategies for repairing damaged myelin in MS.

- The Society launched the Sonya Slifka Longitudinal MS Study. This first study of its kind in the U.S. will collect, on a long-term basis, in-depth information on a national sampling of people with MS in order to address important research questions.

- Society-sponsored researchers at the Mellen MS Center in Cleveland announced results of a study suggesting that individuals with the relapsing-remitting form of MS show progressive loss of brain volume, or atrophy, and that this atrophy may be slowed with interferon beta treatment.

- Investigators at the Weizmann Institute in Israel reported that feeding mice and rats an oral form of Copaxone made their MS-like disease less severe. A large-scale human trial of an oral (pill) form of Copaxone for MS is now being planned. (This drug is currently approved in the U.S. as a daily under-the-skin injection for relapsing-remitting MS.)

- Researchers at Mayo Clinic reported that plasma exchange therapy led to neurologic recovery in about half of 22 people they studied who experienced acute, severe attacks of MS or related disorders and whose neurological deficits were not improved after standard treatment with high-dose steroids.

- As part of the Society's targeted research initiative on gender differences in MS, a small-scale clinical trial of the pregnancy hormone estriol was begun at the University of California at Los Angeles to determine whether it is safe and whether it can control or inhibit MS attacks.

- A small-scale clinical trial of a combination of Avonex and Copaxone in relapsing-remitting MS was begun at 5 centers in the U.S., and an international study was launched to compare the effectiveness of Rebif vs. Avonex in relapsing-remitting MS.

- With Society support, doctors at the University of Southern California began a controlled, Phase 2 clinical trial of "T-cell vaccination" in 80 people with secondary-progressive MS. The "vaccine" is designed to specifically kill immune cells that recognize and launch attacks against myelin insulation in the brain and spinal cord.

- Two experimental treatments were brought before the FDA for approval to treat secondary-progressive MS: Novantrone, a potent immune-suppressing chemotherapy drug; and Betaseron, an immune-system modulator currently approved for relapsing-remitting MS. We should learn in 2000 whether either becomes the first approved treatment for a progressive form of MS.

This fruitful year has brought us closer to achieving our goal: to end the devastating effects of multiple sclerosis.

-- Research Programs Department

EVIDENCE APPENDIX
EXHIBIT 20

"Beyond the Gray Area," Newsday Article, Jamie Talan

Beyond the Gray Area

Study finds generating cells in brain's white matter

By Jamie Talan
STAFF WRITER

WHILE RESEARCHERS revel in the recent news that adult brain cells grow and divide in some areas of the gray matter — the brain tissue where neurons do their work — few people have paid attention to the inner workings of the white matter, the brain tissue covering the gray matter.

Dr. Steven Goldman and his colleagues at Weill Cornell Medical College in Manhattan have evidence of young, dividing cells in the white matter in the adult human brain. The hope is to "mass produce" sufficient numbers of these progenitor, or stem cells, for implantation and cell-based therapies to treat multiple sclerosis, stroke, Tay Sachs and other diseases, Goldman said. The study appears in the Journal of Neuroscience.

White matter is a pool of myelin, or insulation, that surrounds the neurons in the gray matter. It is filled with cells that provide nutritional support to the neurons, including oligodendrocytes and androcytes. The classic lesions identified in patients with multiple sclerosis are found in white matter. Also, one-third of stroke victims suffer lesions in this tissue.

The researchers have developed a method to isolate these progenitor cells by linking them to a gene for a jellyfish fluorescent protein and using a cell-sorting device to separate them from other types of brain cells.

With this pure population of progenitor cells in hand, Goldman said, it would be possible to develop specific techniques to be used after a brain trauma to replace this insulating material and, in theory, fix the brain's transmission problems. (The myelinated axons are the brain's version of telephone wire, long projections that send signals from cell to cell and enable neurons to communicate.)

Goldman, a neurologist, working with his colleague, Neeta Ray, isolated the newly dividing cells from live tissue taken from epilepsy patients and others undergoing brain surgery or biopsy.

Oligodendrocytes are necessary to make myelin, the insulating sheath that surrounds nerve cells that make communication possible. In multiple sclerosis, oligodendrocytes are inflamed or die as the nerve cells' myelin are shredded.

Astrocytes, support cells that provide metabolic support for neurons, are known to grow and di-

vide, but it was always believed that oligodendrocytes, like neurons, do not re-populate. And while scientists have known that the oligodendrocytes are the cells targeted in MS, the immune system disease has always puzzled researchers because the symptoms — tingling in the extremities, tiredness and loss of normal movement — wax and wane. The white matter lesions seem to get better, as well, and it has never been clear how or why axons re-myelinate.

Goldman, a researcher who started his career studying neurogenesis in canary brains, was convinced that there must be a population of oligodendrocyte-progenitor cells that was helping the adult brain heal itself — at least temporarily.

Working side by side with neurosurgeons, Goldman was able to amass snippets of brain tissue from dozens of patients.

By culturing the tissue, the researchers were able to sort out the oligodendrocytes and watch them grow and divide.

In more recent, unpublished work conducted to

Newsday Photo / Patrick Andrade

Dr. Steven Goldman, of the Department of Neurology at Weill Cornell Medical College in Manhattan, studied the brain's white matter.

DID YOU KNOW?

By Kathy Wollard

REMEMBER "You're only as young as you think you are"?

(Left column — partial, edge of adjacent article)

antelope, at the Cincinnati Zoo in 1984, was transferred as an | antelope in an effort to preserve the rare species.

...g vs. Extinction

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coming the problems of breeding in
captivity, including the obvious fact
that in a lab scientists have to deal
with the problem of animals who have
been removed from their natural sur-
roundings.

George Amato of the Wildlife Conser-
vation Society's Science Resource Cen-
ter and the Bronx Zoo in New York
City, said cloning is one tool that can
be used to save rare animals, but he's
not enthusiastic about the method in
his own research.

Amato is using molecular genetic
techniques to help conserve endan-
gered species and, like Dresser, main-
tains a frozen zoo of sorts — a collec-
tion of frozen DNA. Amato said that
when rare animals become difficult to
observe through traditional methods
because they can't be found, DNA
helps the scientists in their research.

"We maintain frozen DNA from ani-
mals from hundreds of species, most of
which are endangered," Amato said.
His frozen DNA collection includes the
the black rhinoceros from Tanzania,
endangered yellow-shouldered Ama-
zon parrots from Venezuela and Ameri-
can crocodiles from Belize.

Despite his reluctance to use clon-
ing, Amato said there is a place for it.
"I do believe it has an application for
very specific cases," he said. "The
panda is probably a reasonable case. I
think that we have some responsibility
to the things living we are driving to
extinction."

Mind Over Brain's White Matter

test whether the cells are function-
al, the investigators implanted the
tissue containing the oligodendro-
cytes into animals with a demyeli-
nating disease.

The new tissue developed into oli-
godendrocytes and made myelin pro-
tein, but whether they line up along
the axon is still not known, Gold-
man said.

"It may one day be possible to acti-
vate a person's endogenous
stem-cell population and generate
these cells on demand," Goldman
said.

Another option, he added, is to pu-
rify these cells and insert them into
the white matter of patients. For ex-
ample, doctors could inject the pro-
genitor-oligodendrocytes into MS
plaques or into the stroke lesions to
trigger re-myelination.

Goldman believes that the popula-
tion of stem cells in white matter
may be far easier to identify, purify
and manipulate than the small
number of progenitor cells found in
the subventricular zone of the gray
matter deep in the brain.

He said there are 10 times as
many white matter support cells,
also called glia, as neurons in the
brain, and that as much as 4 per-
cent of the cells in the white matter
may be progenitor cells.

The neurologist also believes that
it will be possible to insert a single

gene into the progenitor cells, such
as the gene that is abnormal in Tay
Sachs.

This would restore the myelin,
which is damaged and leads to
early death in childhood. Goldman
also believes that stroke will also be
a good model for this modern treat-
ment.

"The possibility to do these experi-
ments is here, now," Goldman said.

He thinks this material could be
in human trials within three years.

"It's one thing to talk about new
brain cells. It's another to be able to
use them for treatment," Goldman
said.

Goldman's group collaborate
with Peter Braun and Michel Graw
el of McGill University in Montreal,
who cloned the oligodendrocyte-spe
cific protein used in the studies to
identify the progenitor cells.

The rush to tap into neurogen
sis — the growth of brain cells — is
fraught with problems.

As it turns out, brain growth isn't
always good, and it isn't always nor
mal. A number of labs are begin
ning to find that growing cells in a
test-tube can lead to a population of
abnormal cells. In other words, the
cells are growing waywardly like
cancers and lose their neuronal
functional capacity.

Goldman's work bypasses this
problem by finding an abundance of
progenitor cells rather than helping
a few cells grow and multiply.

SKY WATCH

ONE OF MY FAVORITE days of
the year is right around the cor-
ner. I'm talking about the first
day of winter. The reason is the winter
solstice, which occurs this year at 2:44
a.m. Dec. 22 on the East Coast. The
winter solstice marks the moment that
the sun reaches its southernmost posi-
tion over our planet and begins its jour-
ney northward. To an observer on
Earth, the day marks the sun's lowest
position in the midday sky, and the be-
ginning of its climb once again.

It all happens because our planet's
equator is tipped by about 23.5 de-
grees to the plane of
our orbit around the
sun. This means
that, during this
time of year, the
Earth's Northern
Hemisphere tilts
away from the sun,
causing the sun's
rays to shine down
on us at a shallow
angle. Six months
and half an orbit
later, our planet's
tilt aims the North-

"solstice" originates in antiquity,
ing from two Latin words —
(meaning "sun") and "sistere" (mean
ing "to stand still").

It is at the winter solstice that
sun's southerly drop seems to end,
sun "stands still," and the star
gives life to planet Earth begins its
cendancy once again. From this mo
ment on, the days become longer,
sun appears higher, and the green
life gradually return to the North
Hemisphere of Earth. And not a
ment too soon, either!

DENNIS MAMMANA / COPLEY NEWS SERVICE
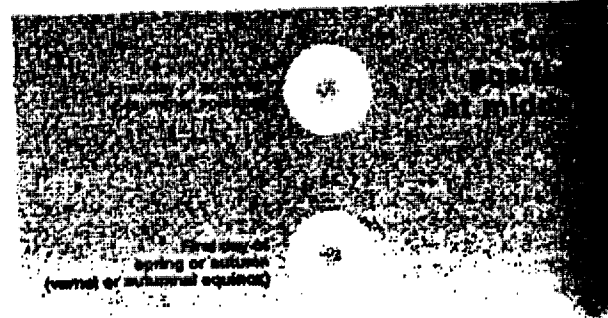
EVIDENCE APPENDIX
EXHIBIT 21

Roy et al., "Identification, Isolation and Promoter-Defined Separation of Mitotic Oligodendrocyte Progenitor Cells from the Adult Human Subcortical White Matter," *J. Neuroscience* 19(22):9986-9995 (1999)

Identification, Isolation, and Promoter-Defined Separation of Mitotic Oligodendrocyte Progenitor Cells from the Adult Human Subcortical White Matter

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Previous studies have suggested the persistence of oligodendrocyte progenitor cells in the adult mammalian subcortical white matter. To identify oligodendrocyte progenitors in the adult human subcortical white matter, we transfected dissociates of capsular white matter with plasmid DNA bearing the gene for green fluorescence protein (hGFP), placed under the control of the human early promoter (P2) for the oligodendrocytic protein cyclic nucleotide phosphodiesterase (P/hCNP2). Within 4 d after transfection with P/hCNP2:hGFP, a discrete population of small, bipolar cells were noted to express GFP. These cells were A2B5-positive (A2B5$^+$), incorporated bromodeoxyuridine in vitro, and constituted <0.5% of all cells. Using fluorescence-activated cell sorting (FACS), the P/hCNP2-driven GFP$^+$ cells were then isolated and enriched to near-purity. In the weeks after FACS, most P/hCNP2:hGFP-sorted cells matured as morphologically and antigenically characteristic oligodendrocytes. Thus, the human subcortical white matter harbors mitotically competent progenitor cells, which give rise primarily to oligodendrocytes in vitro. By using fluorescent transgenes of GFP expressed under the control of an early oligodendrocytic promoter, these oligodendrocyte progenitor cells may be extracted and purified from adult human white matter in sufficient numbers for implantation and cell-based therapy.

Key words: regeneration; myelin; remyelination; cell sorting; stem cells; subependyma

Oligodendrocytes of the adult forebrain are primarily postmitotic. Nonetheless, persistent cycling oligodendrocyte progenitors (OPs) have been described in adult rodent subcortical white matter (Gensert and Goldman, 1996) and may provide a substrate for remyelination after demyelinating injury (Blakemore et al., 1996; Gensert and Goldman, 1997). In humans, the demonstration and identification of persistent subcortical progenitor cells have been more problematic. A pro-oligodendrocytic phenotype has been described in adult human subcortical white matter, although these postmitotic cells may have included mature oligodendrocytes recapitulating their developmental program after dissociation (Armstrong et al., 1992; Gogate et al., 1994). Rare examples of oligodendrocytes derived from mitotic division have been reported in human subcortical dissociates (Scolding et al., 1995), and candidate progenitors have been identified in histological sections on the basis of PDGFα receptor expression (Scolding et al., 1998). Nonetheless, the identification and isolation of viable mitotic oligodendrocyte progenitors from the adult human brain has proven an elusive goal. Indeed, not only have mitotically competent adult human OPs not been preparable in the numbers or purity required for their characterization or functional engraftment, but their very existence in humans has been unclear (Scolding, 1997, 1998).

To establish the existence and relative incidence of oligodendrocyte progenitors in the adult human white matter, we therefore designed a new strategy for the isolation and enrichment of native oligodendrocyte precursors from adult brain tissue. For this purpose, we capitalized on a strategy initially developed for the identification of neuronal precursor cells in which cultured forebrain dissociates were transfected with the gene for green fluorescent protein (hGFP) (Chalfie et al., 1994; Levy et al., 1996), regulated by the early neuronal promoter for Tα1 tubulin (Gloster et al., 1994). This approach permitted the recognition of live, fluorescent neuronal progenitor cells in mixed cell culture. Fluorescence-activated cell sorting (FACS) then permitted the high-yield enrichment and relative purification of these progenitor cells (Goldman et al., 1997; Wang et al., 1998a).

In the present study, we extended this strategy to identify and purify oligodendrocyte progenitors from adult human subcortical white matter. To this end, we used FACS of subcortical cells transfected with hGFP placed under the control of the 5′ regulatory region of an early oligodendrocytic protein, specifically the early promoter (P2) for 2′,3′-cyclic nucleotide 3′-phosphodiesterase (CNP; EC 3.1.4.37) (Vogel et al., 1988; Tsukada and Kurihara, 1992). CNP protein is one of the earliest known myelin-associated proteins to be synthesized in developing oligodendrocytes. It is expressed by newly generated cells of oligodendrocytic lineage, even within the ventricular zone, and appears to be expressed by their precursors as well, in both rodents and humans (Scherer et al., 1994; Yu et al., 1994; Grever et al., 1997; Peyron
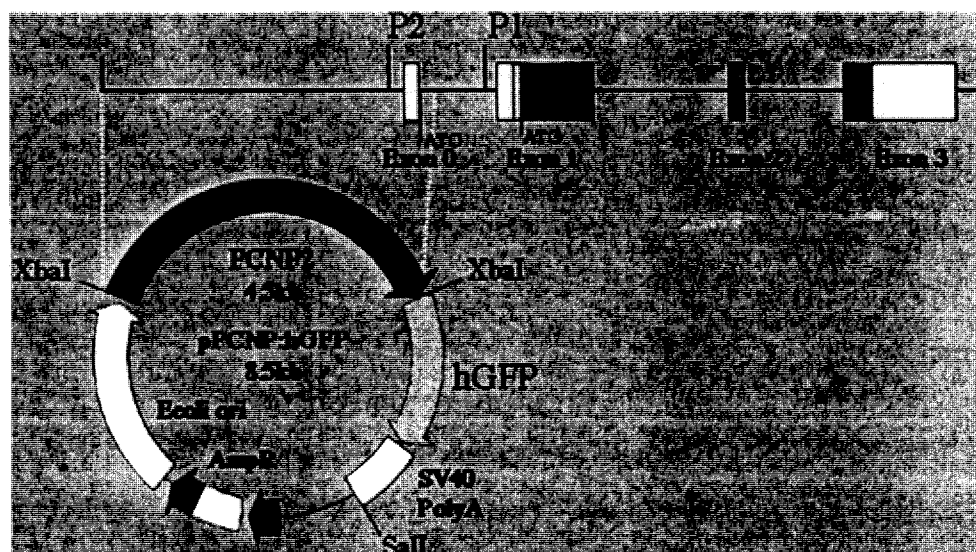
Figure 1. The human P/CNP2:hGFP vector. Humanized GFP (Levy et al., 1996), a mutant form of red-shifted GFP optimized for expression in human cells, was placed under the control of the human CNP2 promoter (P/hCNP2) (Gravel et al., 1996). To construct P/hCNP2:hGFP, an *XbaI-XbaI* fragment encompassing exon 0 and part of intron 1 was isolated from the SK/hgCNP plasmid, which contains the complete sequence of the human CNP gene (Gravel et al., 1996), and was then linked to the hGFP gene fused to the SV40 polyadenylation signal.

et al., 1997; Chandross et al., 1999). Importantly, the 5' regulatory region of the CNP gene includes two distinct promoters, P2 and P1, which are associated with two distinct RNAs, and are sequentially activated at different developmental stages (Douglas et al., 1992; Douglas and Thompson, 1993; Monoh et al., 1993; Scherer et al., 1994) (Fig. 1). Only the CNP mRNA transcribed from the more upstream promoter, P2, is found in the fetal brain, suggesting that the P2 promoter (P/CNP2) directs expression to young oligodendrocytes and their precursors (O'Neill et al., 1997; Gravel et al., 1998). As a result, the CNP2 promoter was chosen for this study for its ability to target transgene expression to oligodendrocyte progenitors and their immature progeny in rodents. On this basis, we postulated that the human homolog of the CNP2 promoter, P/hCNP2, would similarly target transgene expression to human oligodendrocyte progenitor cells.

We report here that the P/hCNP2 has indeed allowed us to direct expression of a reporter gene to oligodendrocyte progenitor cells of the adult human brain and to thereby identify and isolate these cells. Plasmids of hGFP under the control of P/hCNP2, transfected into dissociated subcortical cultures, identified a population of bipolar, primarily A2B5-immunopositive (A2B5$^+$) precursor cells. These cells typically incorporated the mitotic marker bromodeoxyuridine (BrdU) from the culture media and developed oligodendrocytic antigenic expression *in vitro*. Using FACS, we isolated these P/hCNP2:hGFP$^+$ cells from surgically resected subcortical white matter and observed their development into mature, galactocerebroside$^+$ oligodendrocytes in the weeks thereafter. This strategy has allowed us to establish the existence of a distinct class of mitotically competent oligodendrocyte progenitors in the adult human white matter. In addition, P/hCNP2:hGFP-based FACS has enabled us to isolate and separate these cells, viably and in high-yield, and in numbers and purity sufficient to study their cell biology and suitability for engraftment.

Parts of this paper have been published previously in abstract form (Wang et al., 1998b).

MATERIALS AND METHODS

Plasmid construction

P/hCNP2:hGFP and P/hCNP2:lacZ. hGFP, a mutant form of GFP optimized for expression in human cells (Levy, 1996), was placed under the control of the human CNP2 promoter (Douglas et al., 1992; Monoh et

al., 1993; Gravel et al., 1996). The human CNP gene had been isolated previously (Gravel et al., 1996) by screening a human fibroblast genomic library with a cDNA probe for rat CNP1 (Bernier et al., 1987). The human CNP gene was then subcloned into pBluescript, and the resultant plasmid was designated SK/hgCNP. This plasmid was digested with *Bgl*II and *Xho*I to delete much of the gene downstream of the promoter region. The remaining *Bgl*II and *Xho*I ends were then filled in and blunt-end ligated, yielding plasmid SK/P1P2hCNP, in which both *Bgl*II and *Xho*I were regenerated. A 1123 bp *Xho*I-*Xho*I fragment containing SV40 SD/SA-GFPh-SV40 poly(A$^+$) was then excised from pTα1:hGFP (Wang et al., 1998) and subcloned into *Xho*I-digested SK/P1P2hCNP to generate the plasmid P/P1P2hCNP:hGFP. The orientation of the hGFP insert was then determined by restriction enzyme mapping.

To construct P/hCNP2:hGFP, the Tα1 tubulin promoter region was excised from pP/Tα1:hGFP using *Xba*I and replaced with the hCNP2 promoter obtained by digesting SK/hgCNP with *Xba*I. The orientation of the P/hCNP2 insert was also determined by restriction enzyme mapping. Similarly, P/hCNP2:lacZ was constructed by removing the Tα1 promoter from the Tα1:lacZ (Wang et al., 1998a) with *Xba*I, and replacing it with the *Xba*I-*Xba*I fragment containing the hCNP2 promoter. P/CMV:hGFP was constructed as reported previously (Wang et al., 1998a).

Adult human brain white matter dissociation and culture

Adult human brain tissues, obtained freshly in the course of surgical resection, were collected directly into Ca^{2+}/Mg^{2+}-free HBSS. The white matter was dissected from the rest of the tissue, cut into pieces of ~2 mm on edge, or 8 mm^3, and rinsed twice with PIPES solution (in mM: 120 NaCl, 5 KCl, 25 glucose, and 20 PIPES). It was then digested in prewarmed papain–PIPES solution (11.4 U/ml papain; Worthington, Freehold, NJ) and DNase I (10 U/ml; Sigma, St. Louis, MO), on a rocking shaker for 1 hr at 37°C. The tissue was then collected by centrifuging at 200 × g in an IEC Centra-4B centrifuge, resuspended in DMEM-F-12-N2 with DNase I (10 U/ml), and incubated for 15 min at 37°C. The samples were again spun, and their pellets were recovered in 2 ml of DMEM-F-12-N2. They were then dissociated by sequentially triturating for 20, 10, and 5 times, respectively, through three glass Pasteur pipettes fire polished to decreasing bore diameters. Undissociated tissue pieces were eliminated by passage through a fine 40 μm mesh. The cells were collected and rinsed once with DMEM-F-12-N2 containing 20% plasma-derived FBS (PD-FBS; Cocalico Biologicals, Reamstown, PA) to stop the enzymatic dissociation and then resuspended at 1 × 10^7 cells/ml in DMEM-F-12-N2 containing 10% FBS. The cell suspension was plated at 0.1 ml/dish into 35 mm Falcon Primaria plates coated with laminin (2 μg/cm^2) and incubated at 37°C in 5% CO$_2$. After 4 hr, an additional 0.7 ml of DMEM-F-12-N2 with 2% PD-FBS was added into each plate. This medium was supplemented with PDGF AA (20 ng/ml; Sigma), FGF-2 (20 ng/ml; Sigma), NT-3 (20 ng/ml; Regeneron Pharmaceuticals, Tarrytown, NY), and BrdU (10 μg/ml). Cultures were transfected after 2–6 d *in vitro* (DIV). After transfection, the

Figure 2. Adult human white matter harbors oligodendrocyte progenitors. Immunocytochemistry of white matter dissociates for a panel of cell type-selective antigens revealed a diverse representation of phenotypes before sorting. *A–C,* A typical bipolar cell, double-labeled for A2B5 (*red*) and BrdU (*yellow*), fixed after 4 DIV. *D–F.* A cluster of postmitotic O4$^+$ cells (*D, E*) and an overtly less mature BrdU-incorporating O4$^+$/BrdU$^+$ cell (*F*), all fixed after 7 DIV. *G–I,* Representative examples of the diverse phenotypes present in the adult white matter. These included cells expressing CNP (*G*), GFAP (*H*), and TuJ1 (*I*) immunoreactivities, which respectively identify oligodendrocytes, astrocytes, and neurons; each cell type was found in the proportion noted in Results. Scale bar, 40 μm.
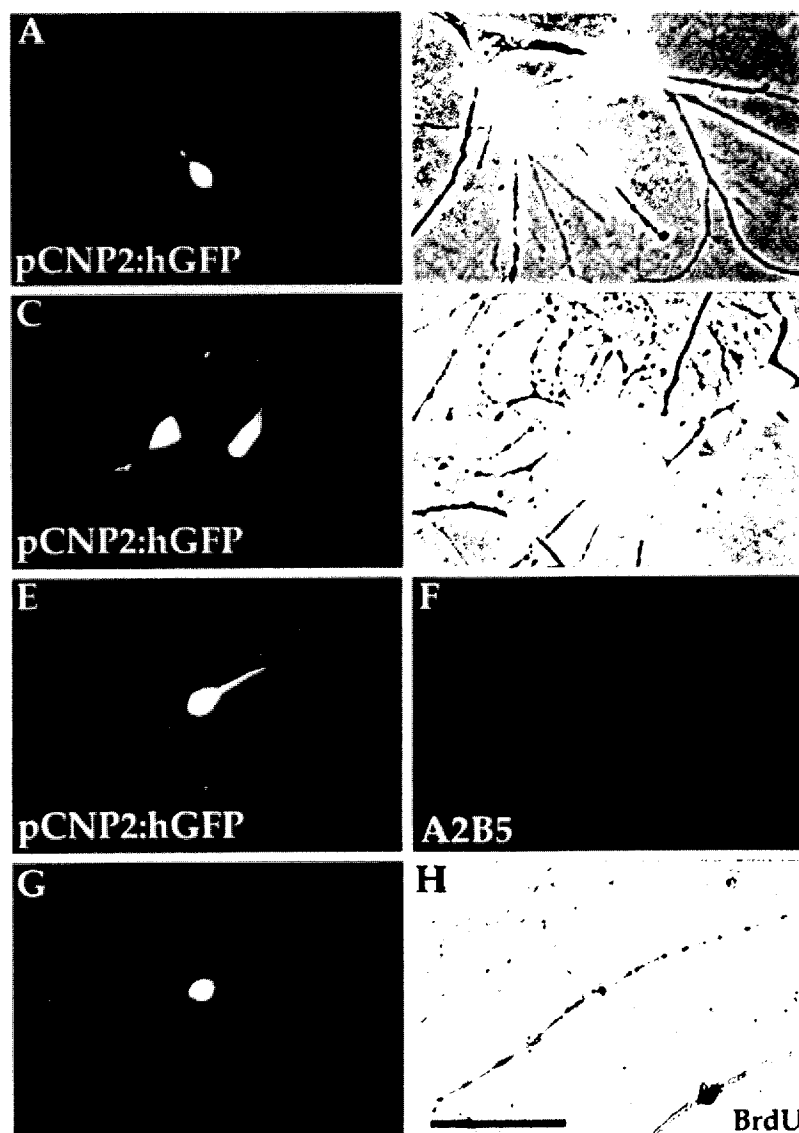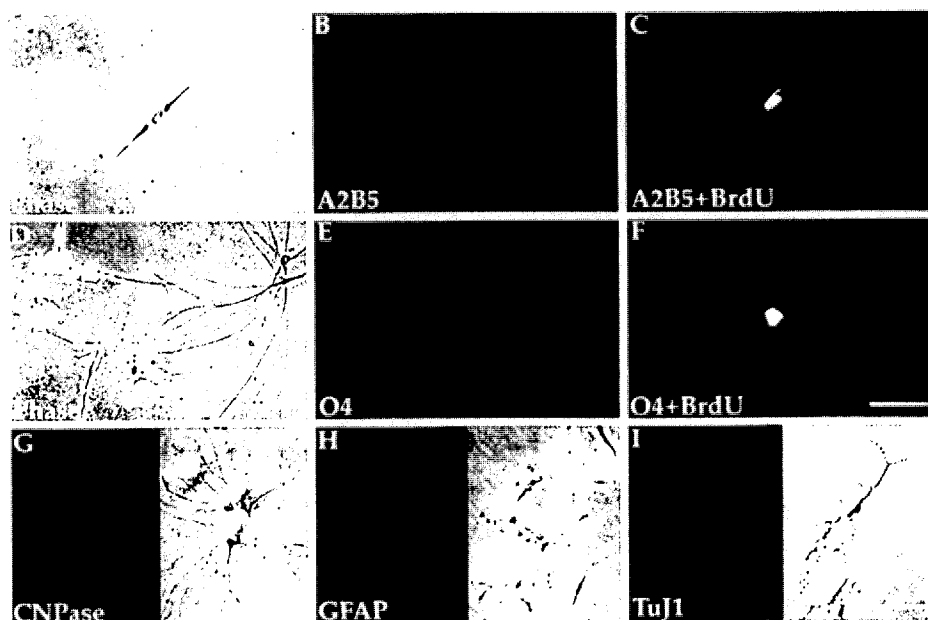

Figure 3. P/hCNP2:hGFP identifies a population of bipolar, A2B5$^+$ cells. GFP expression was observed within 4–5 d after transfection. The P/hCNP2:hGFP$^+$ cells typically first appeared as small, bipolar cells. *A–F,* P/hCNP2:hGFP-expressing cells (*A, C, E*) and their corresponding phase contrast micrographs (*B, D, H*). *E, F,* Immunocytochemistry identified the P/hCNP2:hGFP$^+$ bipolar cells as A2B5$^+$: *G* indicates double-labeling of the two. *Inset* in *H* shows that this cell incorporated BrdU. Scale bar, 30 μm.

cultures were switched to serum-free DMEM–F-12–N2, with maintained growth factor and BrdU supplementation until FACS.

Transfection

All plasmid constructs were introduced into the cultured cells by liposomal transfection, as described previously (Wang et al., 1998a). Briefly, 2–6 d after plating, each 35 mm dish received a mixture of 2 μg of plasmid DNA and 10 μl of lipofectin in OPTI-MEM (Life Technologies, Gaithersburg, MD). The cells were incubated at 37°C in 5% CO_2–95% air for 6 hr. The transfections were terminated with DMEM–F-12–N2 containing 10% PD-FBS. After 2 hr, the cells were returned to serum free DMEM–F-12–N2 with PDGF AA, NT-3, and FGF2. Imaging for hGFP was first done 2 d after transfection and daily thereafter using an Olympus (Tokyo, Japan) IX70 epifluorescence microscope. The greatest number and proportion of GFP $^+$ cells were observed 6–7 d after transfection; cultures were therefore sorted at that time point.

Flow cytometry and sorting

Flow cytometry and sorting of hGFP $^+$ cells was performed on a FACS Vantage (Becton Dickinson, Cockeysville, MD). Cells (5×10^6/ml) were analyzed by light forward and right angle (side) scatter and for GFP fluorescence through a 530 $\pm$ 15 nm bandpass filter as they traversed the beam of an argon ion laser (488 nm, 100 mW). P/hCNP2:lacZ-transfected control cells were used to set the background fluorescence; a false positive rate of 0.02 $\pm$ 0.05% was accepted to ensure an adequate yield. For the test samples transfected with P/hCNP2:hGFP, cells having fluorescence higher than background were sorted at 3000 cells/sec. Sorted cells were plated onto laminin-coated 24-well plates, into DMEM–F-12–N2 containing PDGF-AA, NT-3, and FGF2, each at 20 ng/ml. After 4 d, some plates were fixed for immunocytochemistry, and the remainder was switched to DMEM–F-12–N2 containing 10% PD-FBS. After an additional 3 weeks in vitro, the sorted cells were stained for either CNP, O4, TuJ1, or glial fibrillary acidic protein (GFAP) immunoreactivities; each was double-stained for BrdU as well.

Data analysis

Experimental end points included the proportion of A2B5-, O4-, CNP-, GFA-, and TuJ1-immunoreactive cells in the total sorted population (all nominally GFP $^+$ after sorting), as a function of time after FACS. At each sampled time point, the respective proportions of A2B5 $^+$, O4 $^+$, CNP $^+$, GFA $^+$, and βIII-tubulin/TuJ1 $^+$ cells were compared with each other and with unsorted controls that were similarly dispersed but replated without sorting (after adjusting their cell densities to those of the post-FACS sorted pool). For each combination of treatment (sorted or unsorted), time point (4 d and 3–4 weeks after FACS), and immunolabel (A2B5, O4, CNP, TuJ1, and GFA), the number of stained and unstained cells were counted in 10 randomly chosen fields, in each of three triplicate cultures.

Immunocytochemistry

Cells were immunostained live for A2B5 or O4 (Bansal et al., 1989), or after fixation with 4% paraformaldehyde, for CNP, TuJ1, GFAP, or BrdU. Selected plates were also stained for CD68 or factor VIII, antigenic markers of microglial and endothelial cells, respectively (Kirschenbaum et al., 1994; Rafii et al., 1995). For A2B5 or O4 immunocytochemistry, plates were washed twice with DMEM–F-12–N2 and then blocked with DMEM–F-12–N2 containing 5% normal goat serum (NGS) for 10 min at 4°C. Monoclonal antibody (mAb) A2B5 (clone 105; American Type Culture Collection, Manassas, VA) was used as an undiluted culture supernatant, and mouse mAb O4 (Boehringer Mannheim, Indianapolis, IN) was used at 1:200. Both were applied in DMEM–F-12–N2 for 30 min at 4°C. The plates were then washed with three changes of cold HBSS containing 1% NGS. The secondary antibody, Texas Red-conjugated goat anti-mouse IgM was used at a dilution of 1:50 for 30 min at 4°C. The cells were then washed and fixed with cold 4% paraformaldehyde for 10 min, washed, mounted in SlowFade, and observed using an Olympus IX70 equipped for epifluorescence. Immunocytochemistry for GFAP and TuJ1 was performed according to described methods (Wang et al., 1998a), as was that for TuJ1 and BrdU (Luskin et al., 1997), CD68 (Kirschenbaum et al., 1994), and factor VIII (Leventhal et al., 1999). Selected cultures were also stained for the more mature oligodendrocyte antigens O1 and galactocerebroside, as described previously (Bansal et al., 1989).

RESULTS

Dissociates of adult human white matter harbored a pool of bipolar, A2B5 $^+$ cells

To fully characterize the cell phenotypes resident in adult human white matter, papain dissociates of surgically resected frontal and temporal capsular white matter were obtained from eight patients. These included four males and four females, who ranged from 24 to 65 years old. Three patients had temporal lobe resections for medication refractory epilepsy; two were subjected to decompressive resection during or after extra-axial meningioma removal, two samples were taken during aneurysmal repair, and one was taken from the non-neoplastic approach to a histologically benign ganglioglioma. The monolayer cultures resulting from these white matter dissociations were stained after 5–7 DIV for either of two oligodendrocytic markers, which included the epitopes recognized by the A2B5 and O4 antibodies. Additional, matched cultures were stained after 14 DIV for A2B5, O4, or oligodendrocytic CNP protein, and for either neuronal (βIII-tubulin) or astrocytic (GFAP) target antigens.

In the 14 DIV dissociates of subcortical white matter, 48.2 $\pm$ 10.7% of the plated cells expressed the oligodendrocytic epitope recognized by mAb O4 ($n = 3$ patients, with a total of 935 O4 $^+$ cells among 2041 scored white matter cells; mean $\pm$ SD) (Fig. 2). In matched plates, 49.9 $\pm$ 4.9% were immunoreactive for oligodendrocytic CNP protein, and 7.3 $\pm$ 3.2% expressed astrocytic GFAP. Double-labeling of selected plates revealed that the O4 $^+$ and CNP $^+$ pools were primarily overlapping, with a small proportion of CNP $^+$/O4-negative (O4 $^-$) cells. In contrast, the GFA $^+$ cells only rarely colabeled as O4 $^+$. A small proportion of TuJ1 $^+$ neurons (5.2 $\pm$ 2.2%) was also observed, as were factor VIII-immunoreactive endothelial cells (11.7 $\pm$ 8.9%) and CD68 $^+$ microglia (19.9 $\pm$ 5.5%). Through 30 DIV, the proportions of oligodendrocytes and neurons in these cultures remained approximately stable, with 51.3 $\pm$ 7.0% O4 $^+$ cells and 6.0 $\pm$ 2.1% TuJ1 $^+$ cells, respectively. In contrast, the proportion of GFA-defined astrocytes in these cultures increased from 7.3 $\pm$ 3.2% at 14 DIV to 15.9 $\pm$ 1.4% at 30 DIV ($p < 0.01$ by Student's t test).

Notably, a distinct population of small bipolar cells, which expressed A2B5 but which otherwise expressed neither neuronal nor oligodendrocytic phenotypic markers, was observed; these constituted 1.8 $\pm$ 0.4% ($n = 5$ patients) of all cultured white matter cells at 7 d. However, these cells became scarcer with time in vitro by 30 DIV, and A2B5 $^+$ cells constituted <0.1% of the total cultured cell pool.

The CNP2 promoter targeted GFP expression to a bipolar, A2B5 $^+$ phenotype

To identify either oligodendrocyte progenitor cells or their immature progeny, white matter dissociates were next transfected after 2–6 d with plasmids encoding P/hCNP2:hGFP. Within 4 d after transfection with P/hCNP2:hGFP, a small proportion of GFP $^+$ cells were noted. These were invariably small, bipolar cells and constituted <1% of the total cell pool (Fig. 3). After an additional 4–7 d in vitro, the cultures were immunostained for one of three oligodendrocyte lineage markers, which included A2B5, O4, and CNP protein, or for either astrocytic GFAP or neuronal βIII-tubulin. At that point, the GFP $^+$ cells could generally be described as A2B5 $^+$/O4 $^\pm$/GFAP $^-$/TuJ1 $^-$; 62.5 $\pm$ 8.8% of P/hCNP2:hGFP $^+$ cells expressed A2B5-IR, 21.1 $\pm$ 7.5% were O4 $^+$, and another 7.3 $\pm$ 3.2% expressed astrocytic GFAP. None were recognized by mAb TuJ1, which targets neuronal βIII-tubulin (Menezes and Luskin, 1994). Thus, within the first 7–10

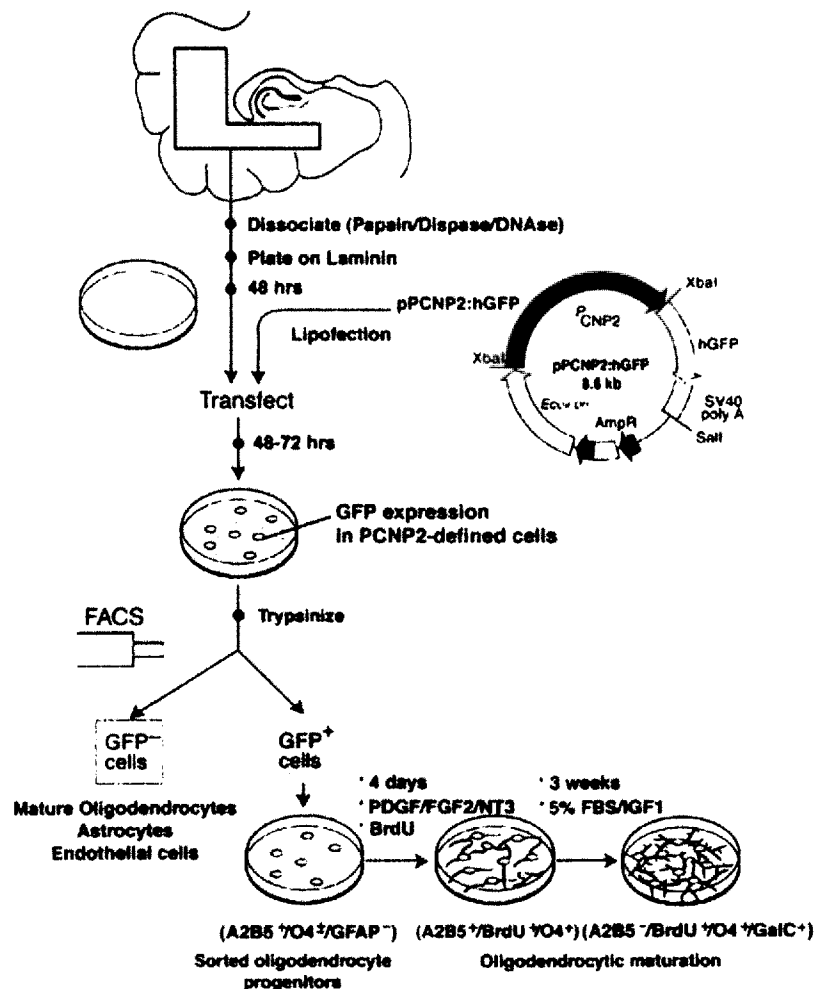# Identification and Enrichment of Oligodendrocyte Progenitor Cells from Adult Human Forebrain

Figure 4. Culture, isolation, and enrichment of oligodendrocyte progenitors. Adult human subcortical white matter, derived from surgical samples of frontal and temporal lobe, was dissected and enzymatically dissociated using papain and DNase and then cultured and transfected with either P/hCNP2:hGFP or control plasmids (P/CMV:hGFP and P/hCNP2:lacZ).

d in culture, P/hCNP2:hGFP selectively identified a population of bipolar, A2B5$^+$ cells. When followed over the weeks thereafter, most of these P/hCNP2:hGFP$^+$ cells developed into oligodendrocytes, which could be recognized by their small, multipolar, heavily branching profiles. Indeed, by 4 weeks, most P/hCNP2:hGFP$^+$ cells expressed O4, whereas only rare cells (<1%) continued to express A2B5 immunoreactivity.

P/hCNP2:hGFP-identified cells were mitotic in vitro

Among white matter dissociates continuously exposed to BrdU and transfected with pP/hCNP2:hGFP on day 4 *in vitro*, 55 ± 14.8% of the resultant P/hCNP2:hGFP$^+$ cells incorporated BrdU by day 7 ($n = 30$ plates, derived from three patients) (Fig. 3). Similarly, 43.1 ± 9.1% ($n = 5$ plates) of the A2B5$^+$ cells in matched plates incorporated BrdU over the same time period. Morphologically, essentially all of these A2B5$^+$ and BrdU$^+$ cells were bipolar at 1 week (Fig. 2). In contrast, the large majority of morphologically mature oligodendrocytes failed to incorporate

BrdU *in vitro*. Only 2.1 ± 1.1% of O4$^+$ cells labeled with BrdU to which they were exposed during the first week in culture, and these few O4$^+$ cells may have just arisen from A2B5$^+$ forebears.

P/hCNP2:hGFP-based FACS yielded a distinct pool of bipolar, A2B5$^+$ progenitors

Using sorting criteria intended for cell type purification, the P/hCNP2-driven GFP$^+$ cells were then enriched and cultured separately (Fig. 4). Immediately after FACS, P/hCNP2:hGFP-separated cells primarily expressed A2B5-IR. Furthermore, the majority of these A2B5$^+$ cells were found to have incorporated BrdU from their culture medium before FACS, indicating their mitogenesis *in vitro* (Fig. 5). Within the week after sorting and with concurrent transfer to higher serum media, most of the sorted cells developed O4 expression and lost A2B5-IR.

Notably, P/hCNP2:hGFP-separable cells were not rare. Among seven patients whose white matter dissociates were trans-
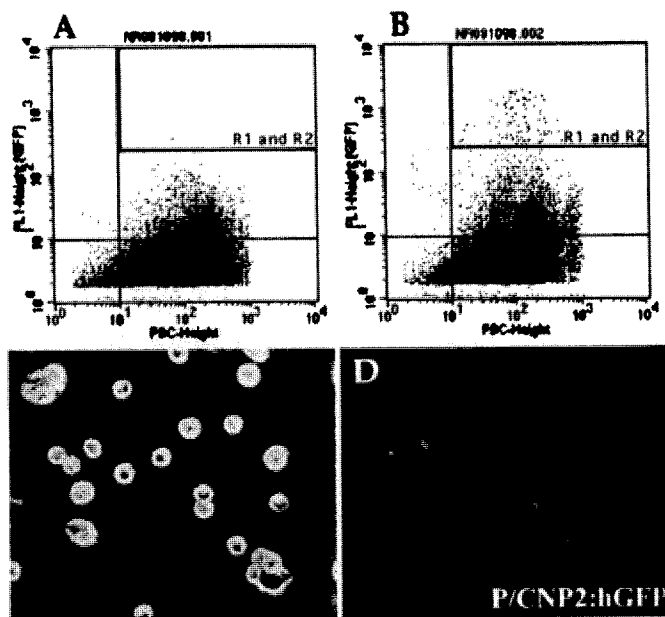
Figure 5. Isolation of P/hCNP2:hGFP⁺ cells by FACS. *A* and *B* shows a representative sort of a human white matter sample, derived from the frontal lobe of a 42-year-old woman during repair of an intracranial aneurysm. This plot shows 50,000 cells (sorting events) with their GFP fluorescence intensity (FL1), plotted against their forward scatter (FSC, a measure of cell size). *A* indicates the plot obtained from a nonfluorescent P/hCNP2:*lacZ*-transfected control, whereas *B* indicates the corresponding result from a matched culture transfected with P/hCNP2:hGFP. The boxed area (*R1* and *R2*) includes those P/hCNP2:hGFP⁺ cells recognized and separated on the basis of their fluorescence emission. The many cells thereby recognized in the P/hCNP2:hGFP-transfected sample (*B*) contrasts to the rare cells so identified in the nonfluorescent P/hCNP2:*lacZ*-transfected control (*A*). *C* and *D* show phase and fluorescence images of GFP⁺ cells 2 hr after sorting. Scale bar, 20 μm.

fected with P/hCNP2:hGFP, 0.59 ± 0.1% of all subcortical cells expressed the transgene and could be separated on that basis. As a result, typically >2000 pCNP2:hGFP⁺ cells (2382 ± 944) were obtained from sorts that averaged 352,000 gated cells (Fig. 6).

Plasmid transfection favored transgene expression by mitotic targets

The incidence of progenitor cells in the adult white matter may be estimated from the frequency of P/hCNP2-defined cells in these cultures, once the transfection efficiency of this cell population is known relative to the overall white matter cell population. In this regard, our net transfection efficiency, determined using P/CMV: hGFP, was 13.5 ± 2.2% (n = 3 plates; 10 low-power fields of each were scored). This suggested that approximately one cell in eight was successfully transfected with the promoter-driven reporter. On this basis, we estimated that oligodendrocyte progenitor cells might comprise as many as 4% (0.59% × 1/0.135 = 4.37%) of all cells in the subcortical white matter. However, this figure needs to be viewed cautiously, because it assumes that all cells in these cultures were transfected and expressed the plasmid vectors with equal efficiency, regardless of their phenotype or mitotic competence. To test this assumption, we exposed a sample of white matter cultures to BrdU, and 3 d later, transfected them with a plasmid of GFP regulated by the constitutively active cytomegalovirus (CMV) promoter (P/CMV:hGFP) (n = 3 plates, with 15 fields from each scored). One week later, the cultures were fixed, and the relative proportions of mitotic (BrdU⁺) and postmitotic (BrdU⁻) GFP⁺ transfectants were determined.

In keeping with the postmitotic nature of mature oligodendrocytes, only 16.1 ± 1.2% of the cells in unsorted white matter cultures had incorporated BrdU by 10 d *in vitro* (n = 20 fields; mean ± SEM). In these same cultures, 9.4 ± 1.0% of the cells expressed GFP placed under the control of the CMV promoter. Remarkably, however, 78.3 ± 6.5% of these GFP⁺ cells were BrdU⁺; this value was over fourfold greater than the BrdU labeling index of the total cell population (p < 0.01 by Fisher's exact test). These data suggested that the transduction efficacy of dividing cells in these cultures was substantially higher than that of postmitotic cells. This in turn suggested that mature oligodendrocytes were either transduced with less efficiency or exhibited less efficient transgene expression than mitotically competent oligodendrocyte progenitor cells. As a result, although the P/CNP2 promoter might have been expected to drive transgene expression in oligodendrocytes as well as their progenitor cells, the greater transfection efficiency of dividing cells would have restricted CNP2:hGFP expression to mitotic cells in the oligodendroglial lineage, resulting in selective GFP expression by the oligodendrocyte progenitor pool. Thus, the enhanced transfection and expression of episomal plasmids in dividing cells, combined with the restriction of P/CNP2 transcriptional activation to oligodendrocyte progenitors and their daughters, appeared to collaborate to account for the selective expression of P/CNP2: hGFP by these adult human oligodendrocyte progenitor cells.

P/hCNP2:hGFP⁺-sorted cells matured primarily, but not exclusively, into oligodendrocytes

Whether mitotic or postmitotic when transfected, the majority of P/hCNP2-sorted cells developed and matured as oligodendrocytes. By 3 weeks after FACS, 74.1 ± 7.7% of these cells expressed oligodendrocytic CNP protein; a matched sample of sorted cells stained after 3 weeks *in vitro* for O4 yielded 66.3 ± 6.8% O4-IR cells, most of which colabeled for the more mature marker galactocerebroside (Fig. 7). Nonetheless, concurrent development of nonoligodendrocytic phenotypes was also noted after FACS purification, albeit at lower frequency than oligodendrocytes; immediately after sorting, 6.5 ± 5.4% of the sorted cells expressed GFAP, and 11.0 ± 4.6% were GFAP⁺ by 3 weeks *in vitro*. These were not simply false positive contaminants because most were observed to express P/hCNP2:hGFP fluorescence. No P/hCNP2:hGFP⁺ neurons, as defined by concurrent TuJ1/βIII-tubulin-IR, were observed immediately before FACS. Surprisingly however, 7.5 ± 4.4% of P/hCNP2:hGFP-sorted cells were noted to mature into βIII-tubulin/TuJ1⁺ neurons in the week after sorting. These TuJ1⁺ cells were similarly confirmed visually as expressing P/hCNP2:hGFP (Fig. 8). Importantly, the presence of these sporadic P/hCNP2:hGFP⁺ neurons and astrocytes after FACS suggests that P/hCNP2-defined progenitors may harbor or retain latent multilineage potential, which may be excercised in the low-density, homogeneous cellular environment of the sorted pool.

DISCUSSION

These data indicate that the adult human subcortex harbors a population of residual, mitotically competent oligodendrocyte progenitor cells. The cells constitute a discrete population of bipolar blasts, distinct from mature oligodendrocytes. The progenitors are mitotically competent, and as such, distinct from the much larger population of mature, apparently postmitotic oligodendrocytes. These cells were antigenically immature (A2B5⁺/O4⁻) when isolated but matured (O4⁺/O1⁺) over several weeks
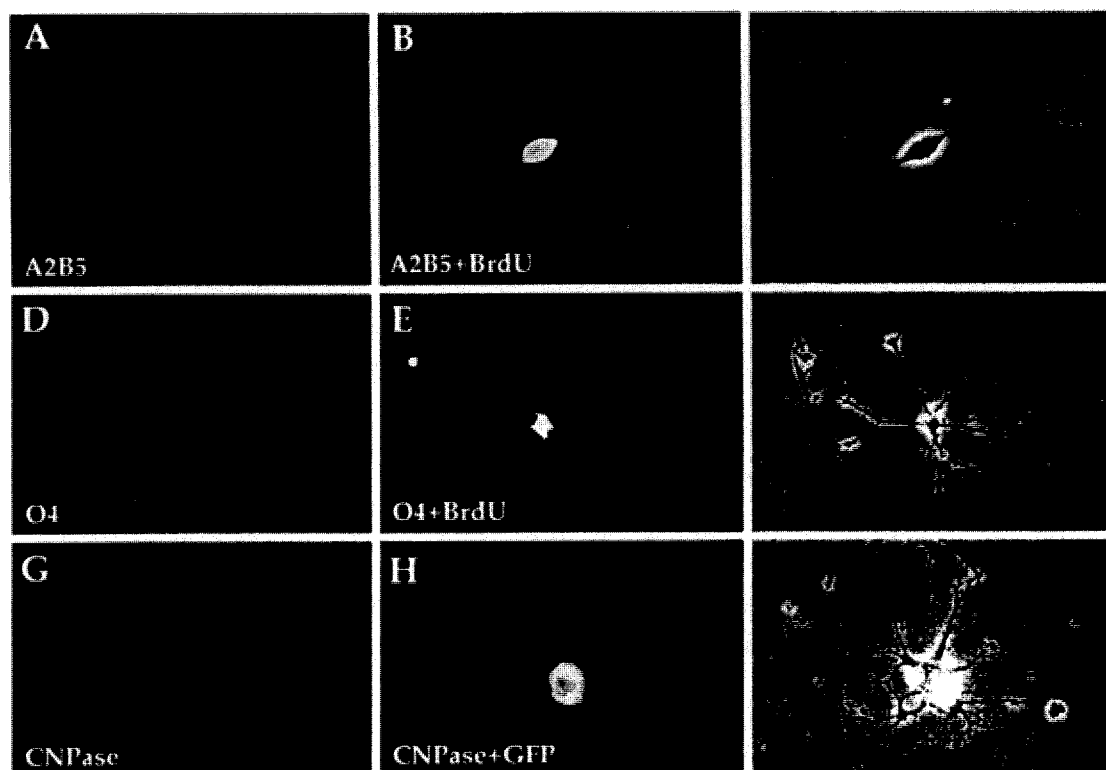
Figure 6. P/hCNP2:hGFP-sorted cells divide and express oligodendrocytic markers. *A–C*, A bipolar A2B5 $^+$/BrdU $^+$ cell 48 hr after FACS. *D–F*, Within 3 weeks, the bipolar cells matured into fibrous, O4 $^+$ cells. These cells often incorporated BrdU, indicating their *in vitro* origin from replicating A2B5 $^+$ cells. *G–I*, A multipolar oligodendrocyte expressing CNP, still expressing GFP 3 weeks after FACS. Scale bar, 20 μm.

in culture. Cell-specific targeted reporting, achieved by transfecting the overall white matter pool with plasmids of GFP placed under the control of the early promoter for oligodendrocytic CNP, allowed the live-cell identification of these progenitor cells. This in turn provided a means for their isolation and purification by fluorescence-activated cell sorting based on P/hCNP2-driven GFP expression.

The nature of the adult white matter progenitor pool

P/hCNP2-defined oligodendrocytic progenitors were not rare. By our sorting criteria, they constituted as many as 4% of cells in the adult human white matter. This figure is marginally greater than previous estimates based on histological identification of PDGFαR expression (Scolding et al., 1998). However, our experiments may have selected for smaller, less fibrous cells, which might be the most capable of surviving tissue dissociation and sorting. Such a bias might have tended to overestimate the incidence of competent progenitor cells in the parenchymal dissociates, so that 4% should be viewed as an upper limit estimate of the incidence of progenitor cells in human subcortical white matter. Nonetheless, the relative abundance and ubiquity of these cells suggest that they may play an important role in the maintenance and function of the normal adult white matter. In rats, a substantial proportion of the white matter cell population is cycling at any one time (Gensert and Goldman, 1996). These cells may be recruited to oligoneogenesis in the event of demyelinating injury (Gensert and Goldman, 1997), and they may be induced to divide *in vitro* by combinations of factors to which they are responsive in development (McMorris and McKinnon, 1996; Shi et al., 1998). Salient differences have been noted in the factor responsiveness of oligodendrocyte progenitors in adult rats and

humans, so that the implications of studies on rodent-derived OPs for human oligodendrocyte progenitor biology remain unclear (Scolding, 1998). Nonetheless, the presence of such a large pool of mitotically competent progenitors in humans suggests that some degree of oligodendrocytic turnover may be occurring in the subcortical white matter. This in turn suggests the possible replacement of damaged or dysfunctional postmitotic oligodendrocytes by progenitor-derived recruits. Recent advances in our understanding of both the humoral and contact-mediated control of oligodendrocyte progenitor expansion in rodents (Shi et al., 1998; Wang et al., 1998c) argue that these endogenous progenitors will prove attractive targets for exogenous activation.

Ontogeny and lineage of parenchymal oligodendrocytic precursors

Neural precursor cells are widespread in the subependymal zone of the forebrain ventricular lining (Goldman and Nottebohm, 1983; Lois and Alvarez-Buylla, 1993; Luskin, 1993; Morshead et al., 1994; Kirschenbaum et al., 1994; Kirschenbaum and Goldman, 1995; Pincus et al., 1998) (for review, see Goldman, 1998; Goldman and Luskin, 1998). At least some of these cells may manifest glial antigenicity *in situ* (Doetsch et al., 1999). Whether the P/hCNP2:hGFP-defined subcortical precursors described here are coderived with the subependymal progenitor pool is unknown. It is also unclear whether the P/hCNP2-defined precursors constitute committed oligodendrocyte precursors or whether they are more intrinsically pluripotential and generate given lineages as a function of the environment to which they are exposed. The latter possibility is suggested by the small proportion of P/hCNP2:hGFP $^+$ cells that were found to be GFAP $^+$
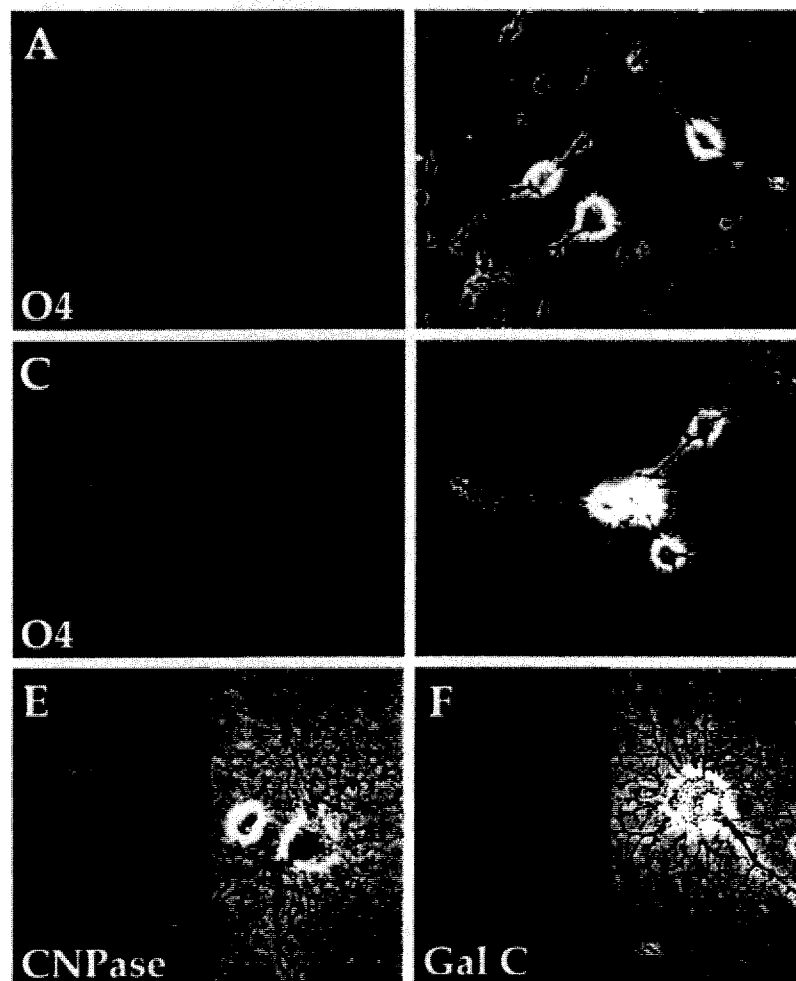
Figure 7. FACS-sorted P/hCNP2:hGFP⁺ cells mature primarily as oligodendrocytes. *A, B,* P/hCNP2:hGFP-sorted cells express O4 (*red*) and begin process elaboration within 4 d after FACS. *C, D,* By 2 weeks after FACS, these cells generally develop multipolar morphologies. *Red,* O4-immunoreactive cells. *E, F,* Progenitor derived-cells matured further over the following weeks, developing oligodendrocytic morphologies and both CNP protein (*E*) and galactocerebroside (*F*) expression by 4 weeks *in vitro.* Scale bar, 30 μm.

astrocytes upon immunostaining; many of these never developed expression of any oligodendrocytic marker and appeared instead to be astrocytes. This suggests that the P/hCNP2-defined progenitor pool may constitute a bipotential astrocyte–oligodendrocyte progenitor, which may yield primarily oligodendrocytic progeny by virtue of the culture conditions we used. As such, this cell type may well be analogous to its A2B5-defined counterparts in

both the perinatal and adult rat optic nerve (Noble et al., 1992; Butt and Ransom, 1993; Colello et al., 1995; Shi et al., 1998).

White matter oligodendrocyte precursors may constitute a pool of multipotential progenitor cells

Whether these cells might also be competent to generate neurons remains unclear. No P/hCNP2:hGFP⁺ cells were found to ex-
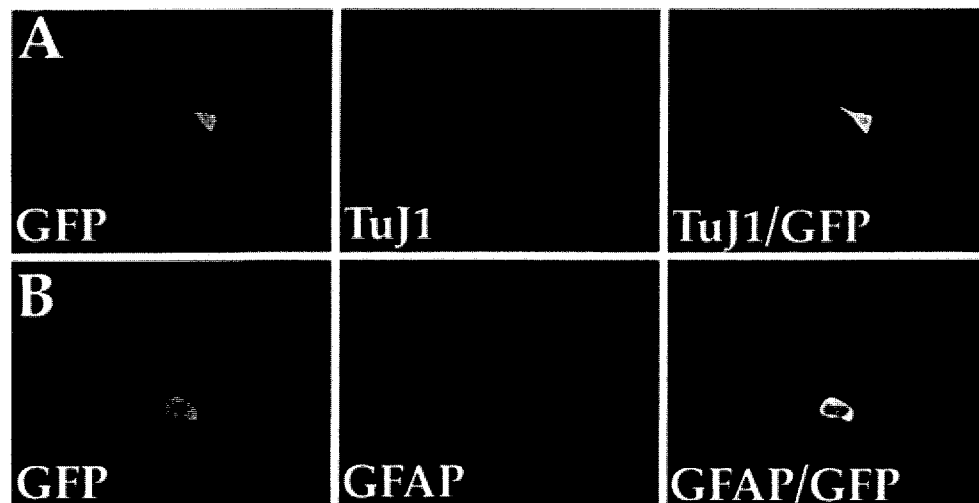

Figure 8. White matter precursor cells may constitute a pool of multipotential progenitors. By 1 week after FACS, some P/hCNP2:hGFP-sorted cells were noted to mature into either TuJ1⁺ neurons (*A*) or GFAP⁺ astrocytes (*B*). Both the TuJ1⁺ (*red* in *A*) and GFAP⁺ (*red* in *B*) cells were confirmed visually as expressing P/hCNP2:hGFP (*green*). No such neuronal differentiation of CNP2:hGFP-identified cells was ever noted in unsorted plates, within which these cells generally matured as oligodendrocytes and much less so as astrocytes. This suggests that P/hCNP2-defined progenitors may retain some degree of multilineage potential, which may be selectively exercised in the low-density, homogeneous environment of a sorted cell pool in which paracrine influences on differentiation are minimized.

press neuronal TuJ1 in unsorted white matter cultures, of >2000 hGFP$^+$ cells studied. Nonetheless, a small number of TuJ1$^+$ cells were noted to develop in P/hCNP2:hGFP-sorted cultures, and these TuJ1-defined neurons were confirmed as P/hCNP2:hGFP$^+$ and were not nonfluorescent contaminants of the sorts. Thus, with time *in vitro*, particularly in the mitogenic FGF2/PDGF/NT3 environment provided here, it remains possible that these cells retain or regain a capacity for multilineage differentiation, as in development (Williams et al., 1991; Davis and Temple, 1994). Importantly, we only noted P/hCNP2:hGFP-defined cells to mature as neurons after high-grade enrichment by sorting. Thus, the multilineage potential of these cells might be preferentially exercised after their isolation from other cell types in low-density culture. As such, the relative fidelity to oligodendrocytic phenotype by P/hCNP2:hGFP-defined cells in the initial white matter dissociates, before FACS, might reflect an initial restriction of progenitor phenotype by paracrine and/or density-dependent influences *in vitro*. Removal and sorting of these cells to low-density, phenotypically homogeneous culture might effectively remove such paracrine restrictions, in essence revealing a multipotential progenitor cell in the adult subcortical parenchyma.

Implantation for the treatment of demyelinating diseases

The high-yield acquisition of oligodendrocyte progenitor cells from the adult human white matter may allow us to better define those growth and differentiation requirements specific to these cells. The potential use of these cells as substrates for induced remyelination, whether upon endogenous activation or engraftment, suggests therapeutic strategies appropriate to a variety of white matter diseases. These potential therapeutic targets include ischemic demyelination, as in subcortical lacunar infarction and hypertensive leukoencephalopathy, postinflammatory demyelinations, such as radiation necrosis and remitted multiple sclerosis, as well as the degenerative and metabolic leukodystrophies.

Together, these observations suggest that a phenotypically distinct pool of oligodendrocyte progenitor cells persists in relative abundance in the adult human white matter. P/hCNP2:hGFP-based FACS permits their viable harvest in sufficient numbers and purity to enable their potential use in cell-based therapeutic strategies.

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EVIDENCE APPENDIX
EXHIBIT 22

Windrem et al., "Progenitor Cells Derived from the Adult Human Subcortical White Matter Disperse and Differentiate as Oligodendrocytes Within Demyelinated Lesions of the Rat Brain," *J. Neurosci. Res.* 69:966-75 (2002)

Progenitor Cells Derived From the Adult Human Subcortical White Matter Disperse and Differentiate as Oligodendrocytes Within Demyelinated Lesions of the Rat Brain

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A distinct population of white matter progenitor cells (WMPCs), competent but not committed to generate oligodendrocytes, remains ubiquitous in the adult human subcortical white matter. These cells are present in both sexes and into senescence and may constitute as much as 4% of the cells of adult human capsular white matter. Transduction of adult human white matter dissociates with plasmids bearing early oligodendrocytic promoters driving fluorescent reporters permits the separation of these cells at high yield and purity, as does separation based on their expression of A2B5 immunoreactivity. Isolates of these cells survive xenograft to lysolecithin-demyelinated brain and migrate rapidly to infiltrate these lesions, without extending into normal white matter. Within several weeks, implanted progenitors mature as oligodendrocytes, and develop myelin-associated antigens. Lentiviral tagging with green fluorescent protein confirmed that A2B5-sorted progenitors develop myelin basic protein expression within regions of demyelination and that they fail to migrate when implanted into normal brain. Adult human white matter progenitor cells can thus disperse widely through regions of experimental demyelination and are able to differentiate as myelinating oligodendrocytes. This being the case, they may constitute appropriate vectors for cell-based remyelination strategies.
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Key words: transplant; remyelination; myelin; precursor cells; neural stem cells; cell sorting

Glial progenitor cells biased toward oligodendrocytic production persist within the adult human white matter and may be isolated and propagated as such (for review see Goldman, 2001). These cells appeared to correspond, in both function and phenotype, to analogous progenitors in the adult rodent white matter, whose phenotypic potential had been previously established in vitro (Wolswijk and Noble, 1989) and validated by retroviral lineage analysis in

vivo (Gensert and Goldman, 1996, 1997; Levison and Goldman, 1999). The existence of postmitotic pro-oligodendrocytes had previously been determined and characterized in the early 1990s by Dubois-Dalq and her colleagues in a series of elegant studies (Armstrong et al., 1992; Gogate et al., 1994), and these cells were subsequently identified and mapped histologically (Scolding et al., 1998). However, mitotically competent progenitors capable of giving rise to oligodendrocytes were not isolated from adult human brain tissue until later (Roy et al., 1999), when promoter-based sorting permitted the isolation of rare or otherwise hard-to-distinguish progenitor cells from native tissues. In this approach, fluorescent reporters such as green fluorescent protein (GFP) are placed under the control of promoters for genes selectively expressed in the progenitor cells of interest (Wang et al., 1998). The chimeric promoter-driven GFP transgenes are then either transfected or infected into the cell population containing the target progenitor cell, and, upon GFP expression by the cells of interest, the progenitor pool is then extracted by fluorescence-activated cell sorting (FACS). This approach has allowed the identification and isolation of rare neural progenitor cell populations from the ventricular zone and hippocampus, from both fetal and

adult human samples (Wang et al., 1998, 2000; Roy et al., 2000a,b; Keyoung et al., 2001).

To determine whether adult human white matter might harbor oligodendrocyte progenitor cells, we constructed plasmid vectors containing the early promoter for the oligodendrocyte protein cyclic nucleotide phosphodiesterase, placed 5' to the coding region for human GFP (hGFP). When we transfected this construct into dissociates of the adult human capsular white matter, we observed that P/CNP2:hGFP was expressed initially by only a single, morphologically and antigenically discrete class of bipolar cells (Roy et al., 1999). These cells were mitotically competent, in that they incorporated bromodeoxyuridine (BrdU) and continued to proliferate in low-serum base medium containing fibroblast growth factor-2 (FGF2), platelet-derived growth factor (PDGF), and neurotrophin-3 (NT-3). Among the P/CNP2:hGFP$^+$ cells, most initially expressed the early oligodendrocytic marker A2B5 but failed to express the more differentiated markers O4, O1, or galactocerebroside. Some expressed astrocytic glial fibrillary acidic protein (GFAP), but none expressed neuronal markers when identified by their GFP fluorescence in mixed, unsorted cultures. When FACS was used to purify these P/CNP2:hGFP$^+$ cells, most were found to mature as oligodendrocytes, progressing through a stereotypic sequence of A2B5, O4, O1, and galactocerebroside expression (Roy et al., 1999), as during development (Noble, 1997). However, occasional neurons were also noted to arise from these cells, particularly in low-density preparations following high-purity FACS, a condition under which sorted progenitors are largely devoid of autocrine and paracrine growth factors. Thus, the nominally glial progenitor of the adult white matter might actually represent a multipotential neural progenitor cell, restricted to the glial and oligodendrocytic lineage by the local white matter environment. As a result, we have designated these cells *white matter progenitor cells* (WMPCs), rather than simply glial progenitors, in recognition of their intrinsically broad lineage potential. Importantly, these cells are not rare: By cytometry based on P/CNP2-driven GFP, WMPCs made up over 0.4% of the sorted white matter cell pool (Roy et al., 1999). With correction for an average plasmid transfection efficiency of 13%, over 3% of dissociated white matter cells might be competent to serve as progenitor cells.

In the present study, we sought to assess the engraftability and myelinogenic competence of these cells when introduced into a region of central demyelination. To this end, we employed a lysolecithin model, in which we injected human WMPCs into the lesioned adult rat brain and then assessed the integration, phenotypic maturation, and myelinogenic competence of the implanted human cells in the environment of the lesioned adult rodent white matter. In addition, we sought to achieve a higher yield means of separating these progenitor cells from surgical samples of the adult white matter, to increase the feasibility of using them in experimental transplantation.

MATERIALS AND METHODS

Adult Human White Matter Dissociation and Culture

Surgically resected adult human brain tissue samples were obtained from five patients ranging from 22 to 49 years old (two males with aneurysms, a female with a geographically distant and histologically circumscribed hemangioma, a female with temporal lobe epilepsy, and a male with an arteriovenous malformation). Surgical resections of forebrain white matter were collected in Ca^{2+}/Mg^{2+}-free Hank's balanced salt solution (HBSS), minced, rinsed twice in PIPES (in mM: 120 NaCl, 5 KCl, 25 glucose, and 20 PIPES), and digested in papain–PIPES (11.4 U/ml papain; Worthington, Freehold, NJ) and DNase I (10 U/ml; Sigma, St. Louis, MO) on a rocker at 37°C for 1.5 hr. The cells were collected by centrifugation at 200g in an IEC Centra-4B centrifuge, resuspended in Dulbecco's modified Eagle's medium (DMEM)/F-12/N2 with DNase I (10 U/ml), and incubated at 37°C for 30 min. The samples were spun again and the pellets recovered in 2 ml of DMEM/F12/N2. The cells were then dissociated by sequentially triturating for 20, 10, and 5 times, respectively, through Pasteur pipettes fire polished to decreasing bore diameters. Undissociated pieces were removed by passage through a 40 μm mesh. The cells were collected and rinsed with DMEM/F-12/N2 containing 10% plasma-derived fetal bovine serum (PD-FBS; Cocalico, Reamstown, PA) to stop the dissociation. The cells were then suspended in DMEM/F12/N2 containing PDGF-AA (20 ng/ml; Sigma), FGF-2 (10 ng/ml; Sigma), and NT-3 (2 ng/ml; Regeneron, Tarrytown, NY) and plated in 100 mm uncoated petri dishes (Corning, Corning, NY).

Magnetic Separation of A2B5$^+$ Cells

After 48 hr in culture, cells dissociated from adult human white matter were collected by washing the plates with Ca^{2+}/Mg^{2+}-free HBSS. The total number of viable cells was determined using calcein (Molecular Probes, Eugene, OR). The cells were incubated with supernatant of hybridoma cells expressing the monoclonal IgM antibody A2B5 (clone 105; American Type Culture Collection, Manassas, VA). Incubation proceeded for 30–45 min at 4°C on a shaker. The cells were washed three times with 10 times the labeling volume in phosphate buffer containing 0.5% bovine serum albumin (BSA) and 2 mM EDTA. The cells were incubated with 1:4 microbead–tagged rat anti-mouse IgM (Miltenyi Biotech) for 30 min at 4°C on a shaker. For flow cytometric analysis, some cells were incubated for the same duration with fluorescein isothiocyanate (FITC)-tagged goat anti-mouse IgM (1:50). The cells were washed three times and resuspended in an appropriate volume of buffer. The A2B5$^+$ cells were separated using positive selection columns, type MS$^+$/RS$^+$ or LS$^+$/VS$^+$ (MACS; Miltenyi Biotech).

Labeling of Human Donor Progenitor Cells

Lipophilic dye tagging. Some A2B5-sorted cells were tagged with 1,1'-dihexadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI; Molecular Probes) at 0.01 mg/ml. DiI-tagged cells were noted to retain their fluorescence discretely for at least 1 week after tagging and transplantation. For longer survival times, cells were identified as donor derived and imaged on the basis of anti-human nuclear antibody immunostaining or by BrdU tagging in vitro prior to implantation, with
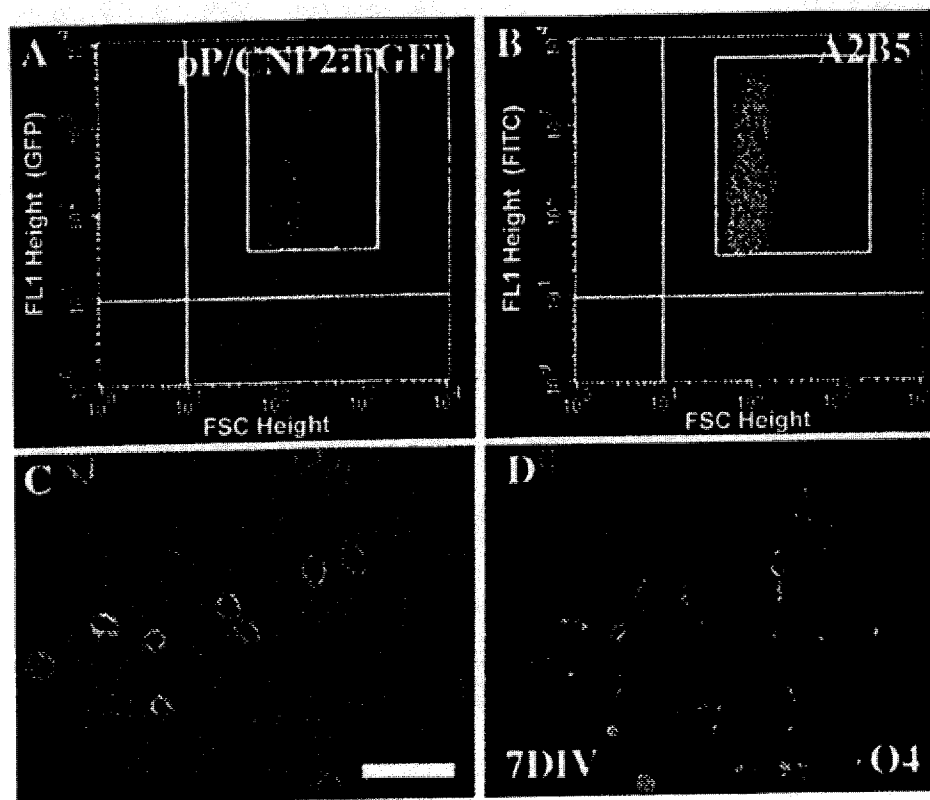
Fig. 1. Oligodendrocyte progenitor cells may be extracted from the adult human white matter. A,B: Separation plots of progenitor cells derived from dissociates of adult human white matter. These graphs plot forward scatter (FCS), an index of cell size, against fluorescence intensity (FL1). A shows separation of progenitors based on CNP2:hGFP, and B exhibits FACS separation based on A2B5-immunoreactivity. The two cell populations sort into homologous quadrants on forward scatter, indicating that the two methods extract cells of the same size. They also sort into identical pools as defined by side scatter (not shown), indicating that their shape and internal reflectance are analogous as well. C,D: By 7 days in culture, most of the A2B5-sorted population had become oligodendrocytes (C, phase; D, fluorescence). This was manifested in their expression of O4 immunoreactivity (green), which recognizes a sulfatide epitope characteristic of oligodendrocytes. Scale bar = 40 μm.

subsequent BrdU immunodetection. Alternatively, some human donor progenitors were genetically tagged with GFP, using lentiviral delivery.

Lentiviral GFP. In some experiments, the A2B5-sorted cells were infected 24 hr after separation with a purified VSV-pseudotyped lentivirus (1×10^5/ml) constructed to express enhanced GFP (EGFP) under the control of the cytomegalovirus (CMV) promoter, with a WPRE 5 woodchuck posttranscription regulatory element. The latter acts like poly-A by stabilizing the transcript without stopping transcription. The lentivirus was generated by cotransfecting plasmids pCMV-D-R8.91, pMD.G, and pHRCMVGFPwsin into 293T cells (Han et al., 1999). Viral particles were collected after 72 hr. The viruses were partially purified by centrifuging the collected supernatant at 60,000g for 2 hr. The sorted A2B5 cells were infected with the virus in the presence of polybrene (8 μg/ml). GFP expression was typically observed by over half of the cells within each infected cell culture within 24 hr after infection. The cells were harvested for transplantation 48 hr after viral infection.

Surgery

Lysolecithin injection. Lesions were produced in the corpus callosa of 200–225 g male rats by stereotaxic bilateral injection of 1 μl of either 1.5% (used for xenografts of GFP-tagged cells only) or 2% lysolecithin type V (Sigma). The coordinates were 1.1 mm posterior to Bregma, 1.0 mm lateral to the midline, and 2.8 mm ventral (Gensert and Goldman, 1997), and the myelinotoxin was delivered at 20 μl/hr.

Transplantation. Three days after lysolecithin instillation, 100,000 A2B5-sorted cells were delivered in 2 μl HBSS into the site of lysolecithin injection, by infusion over 3 min (20 μl/hr). Control animals received comparable injections of saline. Animals were sacrificed at 1 (n = 2), 2 (n = 2), 3 (n = 3), 4 (n = 3), and 8 (n = 1) weeks after xenograft.

Immunosuppression

All animals were immunosuppressed with cyclosporin (Sandimmune; Novartis; 50 mg/ml). Animals received 15 mg/kg daily, beginning on the day of lysolecithin lesion and proceeding daily thereafter until sacrifice. Animals receiving GFP-tagged xenografts were given 20 mg/kg.

Immunohistochemistry

Animals were perfused via intracardiac catheter with HBSS with Ca^{2+}/Mg^{2+}, followed by 4% paraformaldehyde, with postfixation for several hours in 4% paraformaldehyde, passage through increasing concentrations of sucrose to 30%, and freezing during embedding in OCT (Lipshaw). The brains were then cut in 15 μm sections on a Hacker cryostat. Sections were processed for one or more of the following antigenic markers: anti-human nuclear protein (Chemicon, Temecula, CA; MAb1281; 1:50 for 2 days, 4°C), anti-CNP (Sternberger, Baltimore, MD; MAb SMI-91; 1:1,000 overnight, 4°C), anti-human GFAP (SMI 21; 1:1,000, overnight, 4°C), or antimyelin basic protein (anti-MBP; Chemicon Ab980; 1:100 overnight, 4°C). Secondary antibodies included FITC, Cy5, and Texas red-tagged anti-mouse IgM and IgG, anti-rabbit IgG (Jackson
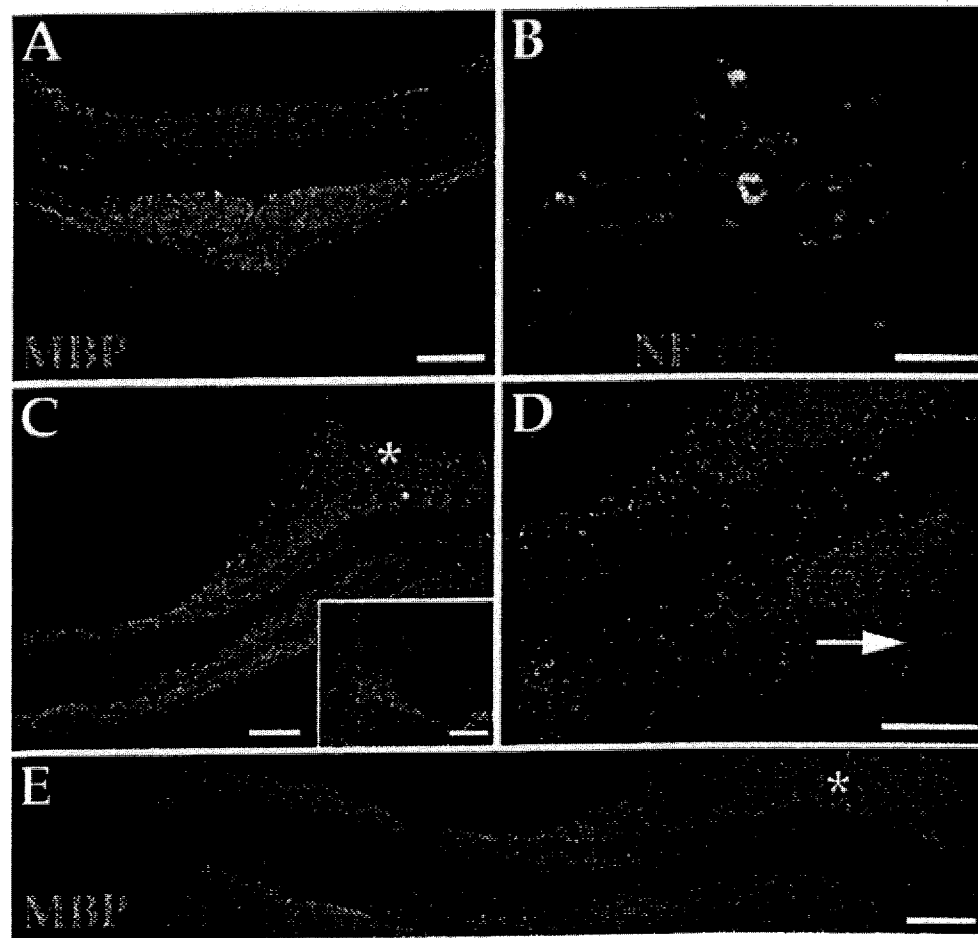
Fig. 2. Implanted white matter progenitors migrated widely throughout the demyelinated callosum. A–E: Sorted adult human white matter progenitors were transplanted into lysolecithin-induced demyelinated lesions in the corpus callosa of adult rats. A shows that lysolecithin infusion yielded demyelinated plaques in the subcortical white matter. This rat was injected with 1 µl of 2% lysolecithin-V, directly into the central core of the corpus callosum, then sacrificed for histology 1 week later and immunostained for myelin basic protein (MBP). The large central lesion is visible as the discoid region of MBP immunonegativity, surrounded by the otherwise MBP⁺ callosum (green). B: Neurofilament⁺ axons (green) initially survived lysolecithin lesion, as seen here 1 week after lesion of the callosum. MBP immunoreactivity (green) has been lost from this lesion core, and implanted progenitors have just immigrated to the lesion (orange). However, axonal spheroids were frequent within the lysolecithin lesion bed, indicating some degree of early injury and transection, to which spheroid formation is a response. The ability of implanted progenitors to effect repair is thus limited by the viability and integrity of the targeted axonal cohort. C: DiI-labeled human progenitor cells (red) 1 week after implant. Even at this early time point, the cells extend throughout the demyelinated lesion, which is characterized by its lack of MBP immunoreactivity (green). The cannula track (*) indicates the site of cell injection into the demyelinated lesion, which was induced 3 days before 10⁵ sorted, DiI-tagged (red) human progenitors were delivered in 2 µl. Inset: Fluorescent microbeads (red) injected into regions of lysolecithin demyelination (MBP; green) failed to disperse beyond their site of injection. D: The transplanted cells migrated throughout the demyelinated plaque, but not beyond its borders, except for occasional migrants that followed the parenchymal surfaces of blood vessels (arrow). The restriction of migration to demyelinated regions suggests that normal myelin impeded the migration of these cells (bottom). E: This low-power montage illustrates the extent and rapidity of migration by engrafted white matter progenitors. Within 1 week of implantation into this demyelinated callosum, the cells traversed the midline to infiltrate the lesion bed in the contralateral hemisphere. The longitudinal extent of this lesion is approximately 6 mm, and the rat was sacrificed 1 week after implantation. Scale bars = 200 µm in A,E; 20 µm in B; 100 µm in C,D; 500 µm in inset.

Immunoresearch, West Grove, PA), and Alexa 488- and 594-tagged anti-mouse and anti-rabbit IgG (Molecular Probes).

Imaging

Brain sections were photographed using an Olympus Fluoview confocal coupled to an IX70 photomicroscope. Images were acquired in both red and green emission channels using an argon-krypton laser, as previously described (Benraiss et al., 2001). The images were then viewed as stacked z-dimension images, both as series of single 0.5 µm optical sections and as merged images thereof. The z-dimension reconstructions were all observed in profile; every human cell doubly labeled with a
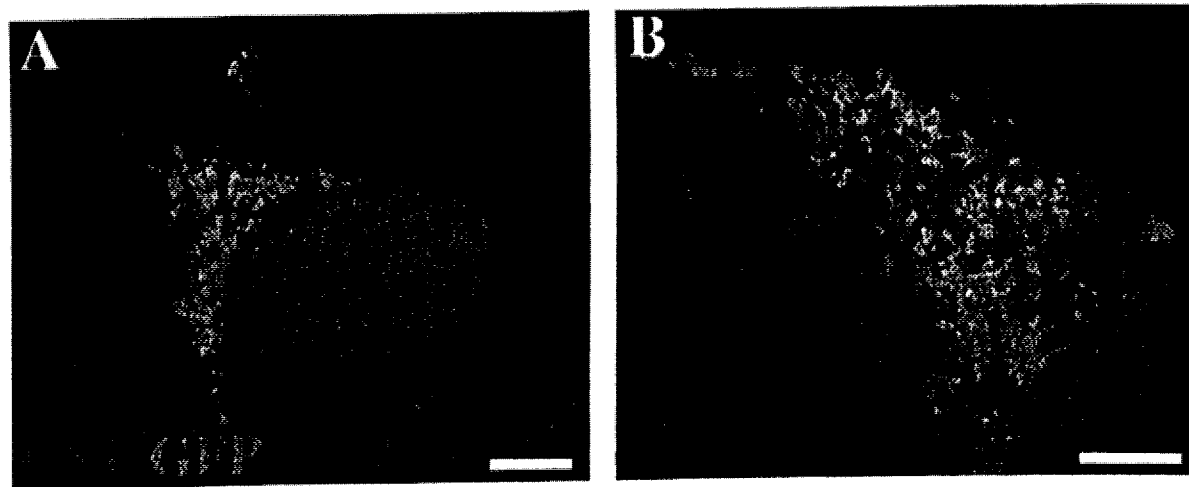
Fig. 3. Normal white matter was nonpermissive for the migration of adult progenitors. A2B5-sorted human progenitors xenografted into the normal adult rat brain failed to migrate beyond the injection site. For this figure, the human cells were prelabeled in vitro with lentiviral GFP (green) to allow the implanted progenitors and their progeny to be identified. A,B: The implanted WMPCs shown in two different fields here were imaged in an animal killed 4 weeks after implantation. The implanted cells did not migrate beyond the borders of the initial injectate. This contrasted with the widespread migration of these cells throughout demyelinated foci, as in Figure 2. Scale bars = 200 μm in A; 100 μm in B.

phenotypic marker was observed orthogonally in both the vertical and the horizontal planes to ensure double labeling.

RESULTS

Oligodendrocyte Progenitors of the Human White Matter Are Selected by A2B5 Expression

In previous studies, we found that transduction of adult human white matter dissociates with plasmids bearing the early oligodendrocytic promoter P/CNP2, driving the fluorescent reporter hGFP, permitted FACS of oligodendrocyte progenitor cells from cultured brain tissue (Roy et al., 1999). Because P/CNP2:hGFP$^+$ cells typically expressed A2B5 immunoreactivity, we asked whether separation based on A2B5 might yield the same pool of mitotic oligodendrocyte progenitor cells. To this end, we first used FACS based on A2B5 expression to extract A2B5$^+$ cells from adult WM dissociates (Fig. 1). We found that 2.7% ± 0.4% of the cells could be separated as A2B5$^+$ (n = 5 patients). This compared with P/CNP2:hGFP-based FACS, from which 0.59% ± 0.1% of the cells could be sorted as P/CNP2:hGFP$^+$; the mean transfection efficiency of 13.5% would have predicted that as many as 4.4% of sorted WM cells were potentially P/CNP2:hGFP$^+$. Using that figure as an arbitrary benchmark, we can estimate that A2B5-based FACS achieved the viable extraction of 57.4% (=2.7/4.4 × 100) of the P/CNP2:hGFP-predicted progenitor cells in the adult white matter.

We next used, based on this figure, immunomagnetic sorting (IMS) to select A2B5$^+$ cells from adult WM dissociates. IMS permits a higher yield than FACS, with a greater recovery and higher viability achieved at the expense of a higher incidence of false positives. We found that, by IMS, 2.87% ± 0.7% of the cells were separated as

A2B5$^+$ (n = 3 patients). This was in accordance with the incidence of WMPCs estimated by both P/CNP2:GFP-based FACS and A2B5-based FACS.

As with the P/CNP2:hGFP$^+$ cells, the A2B5$^+$ cells were mitotic and gave rise largely to oligodendrocytes (Fig. 2). When exposed to BrdU for the first 2 days after sorting, A2B5-defined cells incorporated the label and expanded in number, indicating their persistent replication in vitro. Over the week thereafter, most began to express definitive markers of the oligodendrocytic phenotype; by 1 week after isolation, >70% expressed the oligodendrocytic antigen O4. Together, these data indicate that A2B5-based FACS and IMS of the adult human white matter yields a population of oligodendrocyte progenitor cells that may be homologous to that recognized by P/CNP2:GFP-based isolation and FACS.

Lysolecithin Lesions Provide Demyelinated Foci Appropriate for Experimental Implantation

To establish whether adult human WMPCs could survive xenograft to adult brain parenchyma, we implanted human WMPCs into lysolecithin-demyelinated callosal lesions in adult rats. Lysolecithin is a useful agent for achieving predictable, focal lesions of the white matter (Gensert and Goldman, 1997). It results in local demyelination with local oligodendrocytic loss, some axonal loss, and relative preservation of astrocytic and endothelial elements. Spontaneous remyelination may occur following lysolecithin lesioning and follows a time course that is dependent on the type of lysolecithin (Sedal et al., 1992), its volume and concentration (Woodruff and Franklin, 1999), and the age of the animals (Shields et al., 1999).
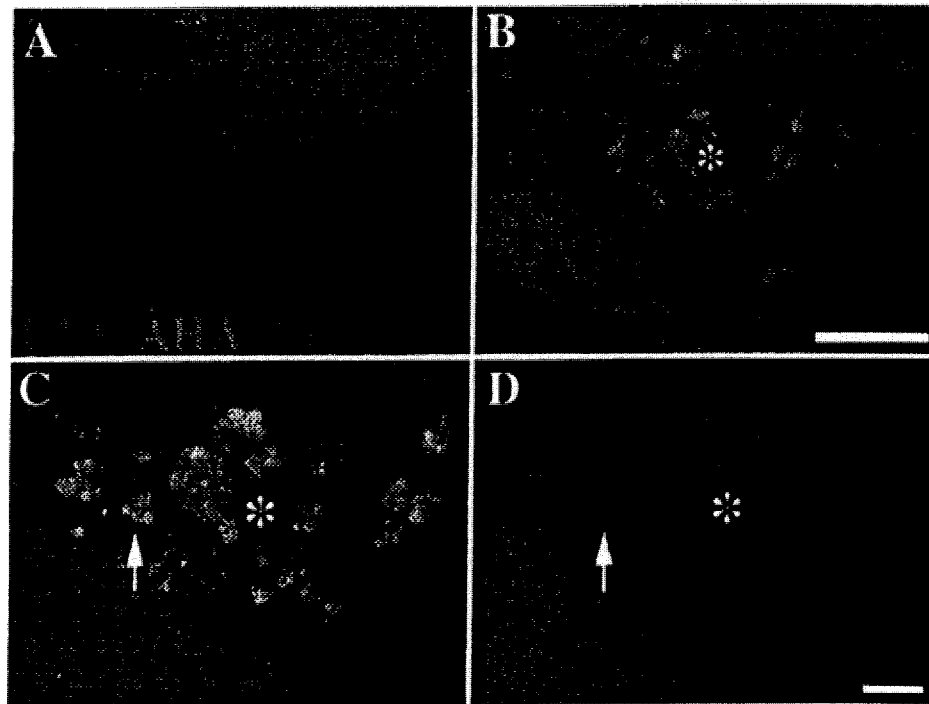
Fig. 4. Xenografted adult white matter progenitors become oligodendrocytes and astrocytes. Implanted A2B5-sorted progenitors typically developed expression of oligodendrocytic CNP protein within 2 weeks of implantation. A: In a control animal that received a saline injection, CNP protein (red) remains absent from the demyelinated central core of the callosum (black) 15 days after lesion. In the matched animal shown in B, human white matter progenitor cells, recognized by anti-human nuclear antibody (AHA; green), were noted both to fill in the lesion and to express oligodendrocytic CNP (red) 15 days after implantation. C shows a higher magnification of this field; D demonstrates CNP expression (red) associated with the cell bodies of the human cells visualized as AHA$^+$ in B. Asterisks are located in the same position; arrows in C and D indicate the same cell. Scale bar = 20 μm in B (for A,B); 5 μm in D (for C,D).

Therefore, lysolecithin lesions mimic salient aspects of acute inflammatory demyelination.

We first confirmed the prior observations of Gensert and Goldman (1997) that lysolecithin lesion was associated with a focal lesion of capsular myelin, with initial injury predominantly limited to oligodendrocytes and their myelin. When assessed 1 and 3 weeks after 1 μl injections of 2% lysolecithin-V, these lesions exhibited a mild degree of reactive astrocytosis within the demyelinated focus, the vascular architecture of which appeared intact. No myelin could be visualized by staining for MBP within 2 mm of the callosal injection site. In addition, oligodendrocytes were markedly diminished, with a >95% loss of CNP$^+$ cells within the MBP-demarcated lesion (Fig. 2A). Axons were present, as assessed by neurofilament staining, but axonal spheroids were common, indicating some degree of axonal damage and early loss (Fig. 2B).

Adult Human-Derived A2B5-Defined Progenitors Survive and Rapidly Migrate Upon Xenograft to Lysolecithin-Demyelinated Foci of the Adult Rat Brain

We next prepared A2B5-sorted progenitor cell pools from adult human white matter and stereotaxically im-

planted them into both normal and lysolecithin-lesioned adult rat brain. A2B5$^+$ cells (1 × 10^5) were implanted into each lesion bed 3 days after a 1 μl injection of 2% lysolecithin. Some donor cells were prelabeled with the lipophilic tracking dye PKH26 to allow their detection after implantation (Horan and Slezak, 1989). Other donor cells were instead localized using human-specific donor cell antigens. At 1, 2, 3, 4, and 8 weeks after implantation, the recipient brains were fixed and prepared for histologic analysis.

The implanted cells migrated rapidly, throughout the extent of the demyelinated lesions. Within 1 week of implantation, the cells readily traversed the midline to infiltrate the farthest reaches of the demyelinated lesion beds, which often extended over 6 mm in breadth. The migration rate of the cells was hence roughly 1 mm/day, or almost 50 μm/hr within the lesion (Fig. 2B–D).

The surprisingly rapid and extensive migration of the implanted cells raised the possibility that the initial pressure of the injection was contributing to their local dispersal. To ensure that implanted donor progenitor cells were not infiltrating their target lesions as a function of hydraulic pressure, we slowly infused them in 2 μl over 3 min. As an
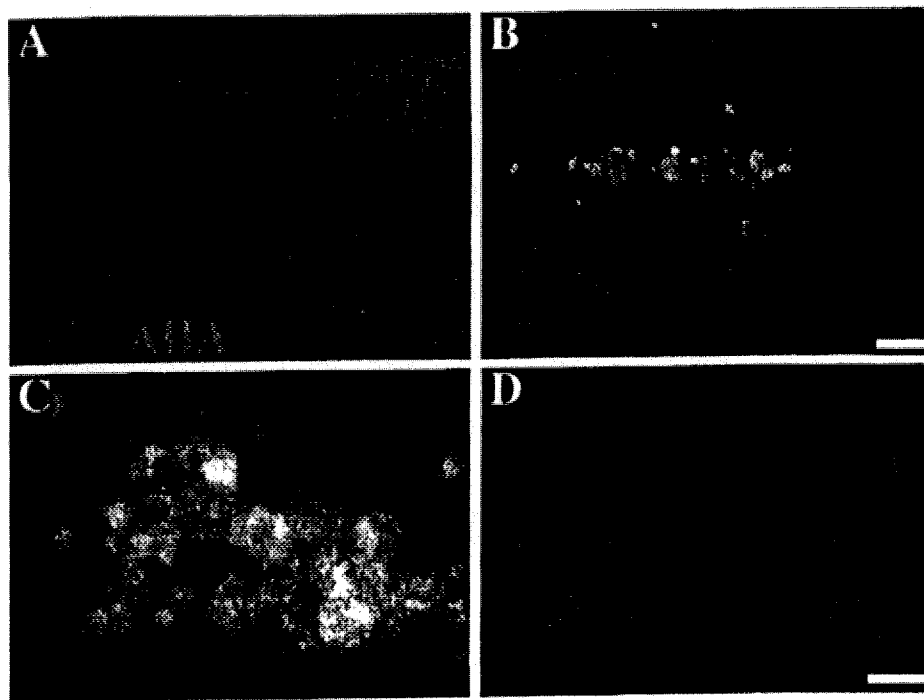
Fig. 5. White matter progenitors express MBP after engraftment to demyelinated foci. Sorted oligodendrocyte progenitor cells derived from the adult human white matter were injected into adult lysolecithin-lesioned rat corpus callosum and were noted to express MBP within 3 weeks thereafter. A: In a control animal that received a saline injection, MBP (red) remains absent from the demyelinated central core of the callosum (black) 3 weeks after lesion. B: In a rat that received 100,000 sorted progenitor cells unilaterally, human nuclei (green) are surrounded by MBP (red) in the center of the lesion 21 days after implantation (24 days after lysolecithin injection). C,D: High-power image showing a cluster of AHA$^+$ human cells (green) associated with a plethora of MBP$^+$, myelinating, oligodendrocytic membranes (red). D focuses on the MBP$^+$ membranes of this field; the oligodendrocytic lamellopodia (red) appear in various stages of ensheathment. Scale bar = 20 μm in B (for A,B); = 5 μm in D (for C,D).

additional control, two rats were injected with 6 μm fluorescent microbeads (Becton-Dickinson, San Jose, CA; 488 nm excitation). In total 100,000 beads (2 μl/3 min) were delivered into callosal foci of lysolecithin demyelination 3 days after lysolecithin injection. The rats were sacrificed 2 hr after surgery, and their brains were cryosectioned and immunostained for MBP. The microbeads were noted to line the cannula track and otherwise remained within a focal deposit at the injection site (Fig. 2C). These findings strongly suggested that pressure injection per se was not associated with significant mechanical dispersion of injected cells.

Normal Brain Is Nonpermissive for Migration of Adult Oligodendrocyte Progenitor Cells

Despite the rapid migration of the implanted progenitor cells throughout the lesion beds (Fig. 2B,C), the cells were typically restricted to regions of demyelination, rarely extending into normal surrounding myelin. Even the few cells that were typically noted to have infiltrated normal myelin appeared to have migrated therein along the extraluminal surfaces of blood vessels. The latter appeared to be limited to vessels that at some point traversed the lesion bed and thereby presented their adventitial surfaces to the migrating implanted progenitors.

On this basis, we asked whether the restriction of implanted progenitors to the lesion site reflected a relative preference for the implanted progenitors to the demyelinated lesion site or whether it instead reflected an absolute impediment of normal white matter to progenitor migration (Jefferson et al., 1997). To this end, we genetically tagged A2B5-sorted adult human WMPCs with CMV-driven EGFP by infecting them in vitro with a lentiviral GFP vector (see Materials and Methods). The fluorescent human WMPCs were then implanted into the intact subcortical white matter of four adult rats to loci including the callosum, hippocampal commissure, and stria medullaris. The fate of the tagged cells was then assessed by sacrificing three of the animals 1 month after implantation and one rat at 2 months. We found that, when injected into intact white matter, the adult progenitor cells remained localized to the implant sites: Whether assessed 4 or 8 weeks after implantation, the cells migrated no farther than the bounds of the initial injectates (Fig. 3).

Adult White Matter Progenitors Differentiate as MBP$^+$ Oligodendrocytes Upon Xenografting

The engraftment sites each harbored substantial populations of viable cells, many of which expressed CNP protein, indicating their oligodendrocytic maturation.
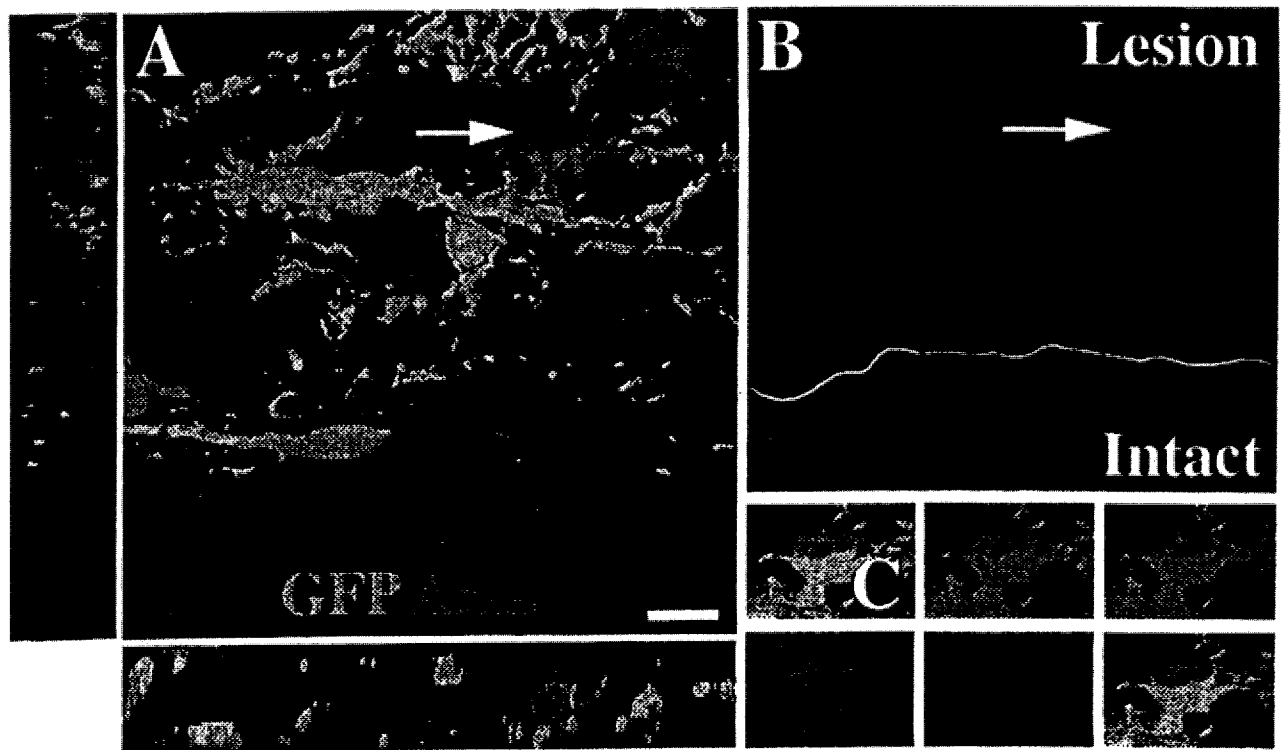
Fig. 6. Genetically tagged adult human-derived white matter progenitors can mature and survive after xenografting. Sorted white matter progenitor cells were tagged with lentivirally delivered GFP, then injected into the lysolecithin-lesioned rat corpus callosum. The recipient animal was killed and its brain immunostained and imaged by confocal microscopy 8 weeks after cell implantation. In A, the GFP$^+$ human progenitors (green) are seen to express human nuclear antigen (AHA; orange), confirming the stability and donor cell restriction of the tag. The side and bottom panels appended to A show orthogonal side views taken through the indicated MBP$^+$ human donor-derived cell (blue/green; arrow). B: Blue color channel of A shows that several of the GFP-tagged human progenitors implanted into the lesion site have differentiated as MBP$^+$ oligodendroglia (line demarcates lesion border). Many have also matured as astrocytes (data not shown). C shows the cell indicated in A, emphasizing its coexpression of lentiviral GFP, human nuclear antigen (AHA), and MBP. Scale bar = 20 μm.

CNP expression typically appeared in implanted adult A2B5-sorted progenitors within 2 weeks of implantation (Fig. 4). By 3 weeks, many had developed expression of MBP (Fig. 5). These human donor-derived cells were noted to project MBP$^+$ lamellopodia; at low magnification, they were associated with a fine, filamentous array of myelinating fibers. These observations suggested the initiation of progenitor-associated myelinogenesis within the lesion site (Fig. 5). With cyclosporin immunosuppression, we found that these cells could survive for at least 2 months in lysolecithin-demyelinated rat recipients (Figs. 6, 7).

To visualize better the expression of myelin-associated antigens by implanted human progenitors, we also implanted four lysolecithin-lesion animals with lentiviral GFP-tagged human WMPCs. These animals were sacrificed after 4 or 8 weeks, and the fate of the tagged progenitors was assessed histologically. At both time points, GFP-tagged cells were found to have differentiated as admixed populations of oligodendrocytes and astrocytes. No neurons were noted to have arisen from these engrafted progenitors in a matched set of βIII-tubulin-immunostained sections (not shown). Within the lesions,

many MBP$^+$ oligodendrocytes were noted to be GFP tagged and, hence, derived from donor human progenitor cells (Fig. 6). At the lesion borders, a preponderance of GFAP$^+$ GFP-tagged cells was typically noted, indicating the astrocytic differentiation of many of the implanted progenitors (Fig. 7). Few cells of either type migrated beyond the lesion borders.

DISCUSSION

Our previous studies revealed the existence of a distinct population of mitotic oligodendrocyte progenitor cells in the adult human subcortical white matter. These cells are present in both sexes and into senescence and are both ubiquitous and relatively abundant in the adult forebrain white matter. In this study, we found that these oligodendrocyte progenitor cells of the adult human subcortical white matter may also be identified and isolated on the basis of their surface expression of the epitope recognized by MAb A2B5 and that this antigenic phenotype includes those cells defined by CNP2-driven GFP. IMS based on A2B5 expression has allowed us to extract these
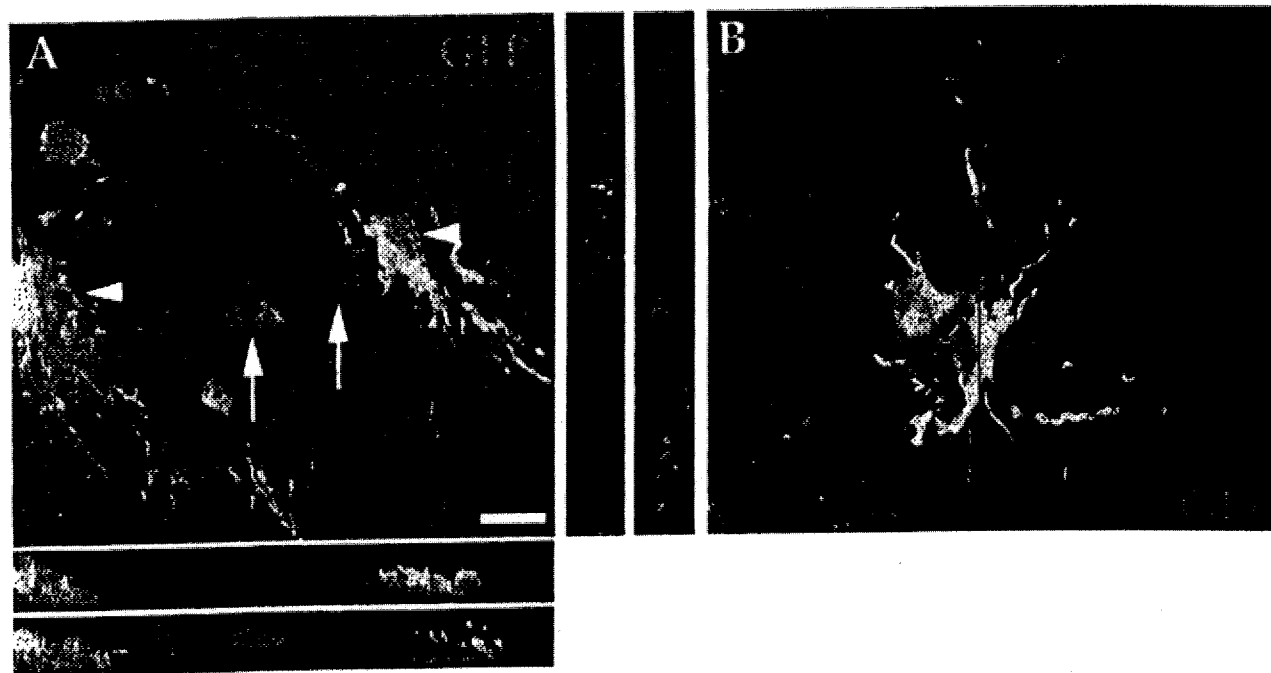
Fig. 7. Both astrocytes and oligodendrocytes arose from implanted adult human white matter progenitors. In A, a confocal composite shows two GFP-tagged human (green) MBP⁺ (red) oligodendrocytes in the lesion bed of a lysolecithin-injected rat callosum 8 weeks after cell implantation. In addition to the MBP⁺ cells (arrows), other human progenitor-derived cells were also present that did not express MBP and that instead manifested astrocytic morphologies (arrowheads). In B, immunolabeling for human GFAP (red) revealed that many of the GFP-tagged human progenitors had in fact given rise to astrocytes. Scale bar = 20 μm.

cells from human white matter dissociates in numbers sufficient for experimental xenografting.

The implanted white matter progenitors were found to be highly motile and migratory, infiltrating the demyelinated regions of the white matter over distances up to 1 cm in the week after implantation. However, they were found to avoid normal myelin, which effectively excluded their infiltration. The failure of WMPCs implanted into the normal brain to migrate beyond the injection bed stood in sharp contrast to the fate of otherwise identical cells implanted into lesioned white matter, which migrated rapidly and efficiently throughout the available demyelinated lesion bed. These observations suggest that normal adult white matter is nonpermissive for the migration of adult-derived WMPCs. This restriction appears to be stringent and does not merely reflect demyelinated tissue acting as a preferential substrate for progenitor migration. In a general sense, progenitor cells may be subject to the same types of negative influences on their migration as are axons, whose extension is suppressed in the environment of normal white matter (GrandPre et al., 2000; Chen et al., 2000). However, whereas several myelin-associated moieties that suppress axonal extension, and the axonal receptors for these repulsive ligands, have been identified (Fournier et al., 2001), the operative white matter signals that restrict progenitor cell migration have yet to be determined. The characterization of these repulsive ligands and of their anticipated progenitor cell receptors will likely constitute an important avenue for future study.

The engrafted adult-derived progenitors differentiated largely as oligodendrocytes, and also as astrocytes, and exhibited myelin protein expression in regions of experimental demyelination. The time course of this process was relatively rapid; oligodendrocytic differentiation, as reflected by CNP protein expression, ensued within 2 weeks of donor cell isolation and implantation. Myelinogenesis appeared to follow closely, such that MBP expression attributable to donor cells was evident within 3 weeks of implantation. The efficiency of myelination was difficult to assess in this study, insofar as we did not systematically assess the persistence of axons in these lesions. This caveat notwithstanding, our observations suggest that the introduction of highly enriched preparations of progenitor cells derived from the adult human white matter may permit the structural repair of demyelinated lesions in the adult CNS.

ACKNOWLEDGMENT

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